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Award Number: DAMD17-00-1-0032

TITLE: Intercellular Communication and Chemoprevention of Prostate Cancer by Analogs of Vitamin A and D

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REPORT DATE: January 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2002	Annual (1 Jan 00 - 31 Dec 01)

4. TITLE AND SUBTITLE
Intercellular Communication and Chemoprevention of Prostate Cancer by Analogs of Vitamin A and D

5. FUNDING NUMBERS
DAMD17-00-1-0032

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Though the natural and synthetic analogs of vitamin A and D are used as chemopreventive agents, their use in clinical trials has been hampered due to toxic effects. We have identified 2 genes that regulate the invasiveness of prostate cancer (PCA) cells. They code for proteins called connexins which assemble to form specialized structures called gap junctions that allow the exchange of small molecules between cells— a phenomenon called intercellular communication. Our studies show loss of connexins in advanced PCA, and reintroduction of connexins corrects the invasiveness of PCA cells. Our studies further show that treatment of human PCA cell lines with vitamin A and D analogs enhances gap junction formation. We have proposed that strategies designed to direct chemopreventive agents to restore defective intercellular communication in prostate tumors may prove to be more effective. To reduce the side effects associated with vitamin A and D analogs, we have proposed experiments that will help us optimize concentrations of these agents, either alone or in combination, that will be the least toxic and most effective in restoring defective communication in PCA cells. We anticipate that these optimized concentrations will also be the most effective in the chemoprevention of PCA.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Prostate Cancer	82
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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1. Introduction

Gap junctions, formed of proteins called connexin(Cx)s, provide a direct intercellular pathway for the passage of small signaling molecules between the cytoplasmic interiors of adjoining cells. It has been proposed that alterations in the expression and function of connexins may be one of the genetic or epigenetic changes involved in the initiation and progression of neoplasia (1-4). Our studies have shown that Cx expression in epithelial cells of prostate tumors is lost during progression of prostate cancer (PCA), with a severe loss in its advances stages. We have also shown that forced expression of Cx32 and Cx43 — the 2 Cxs expressed by the well-differentiated epithelial cells of the prostate — into Cx-deficient, indolent, and androgen-dependent human PCA cell line (LNCaP) resulted in the formation of abundant gap junctions, enhanced communication, inhibited growth in vivo and in vitro, and induced differentiation (5,6), whereas that into invasive and androgen-independent PCA cell line (PC-3) resulted in the cytoplasmic accumulation of connexins(7-10). Treatment of Cx-expressing LNCaP clones with all-trans-retinoic acid (ATRA) and 1α, 25 dihydroxyvitamin D_3 (1,25 D) enhances the assembly of Cxs into gap junctions as we had observed in our earlier studies with a different system (2,3). The 2 objectives of our proposed study are to:

(1) Optimize concentrations of vitamin A and D analogs (either alone or in combination) that are most effective in facilitating the assembly of connexin32 and connexin43into gap junctions using human prostate cancer cell lines expressing these connexins constitutively or inducibly.

(2) To test whether these optimized concentrations of vitamin A and D analogs are also the most efficacious in inhibiting the growth and inducing the differentiation and apoptosis of human prostate cancer cell lines expressing connexins constitutively or inducibly in vitro.

2. Body

Task 1

The aim of task 1 was to construct doxycycline/tetracycline (Dox/Tc)-responsive retroviral vectors harboring Cx32 and Cx43 cDNAs and generate packaging cell lines producing recombinant retroviruses that, upon infection, are able to transduce these Cxs into androgen-dependent(LNCaP) and -independent (PC-3) human prostate cancer cell lines.

For reasons mentioned in pages 4 and 5 of previous report, the objectives of Task 1 could not be accomplished becauseDox/Tc-inducible cassette contained in a single retroviral vector (pTet-Off and pTet-On) could not be used due to the massive rearrangement of connexin cDNA fragments (pages 4-6 of previous report). Thus although the objectives of task 1 were technically fulfilled, Cx-harboring retroviruses could not be successfully used to achieve task 2, which we had proposed to accomplish in a different way.

Task 2

The aim of task 2 was to characterize 3 clones derived from each of LNCaP and PC-3 cell lines in which the expression of Cx32 and Cx43 could be induced 10-1000 fold (by the addition of Dox/Tc) and ascertain if inducible expression of these Cxs affects their growth, differentiation and apoptosis similar to (already characterized) LNCaP and PC-3 clones expressing these Cxs constitutively.

Because Dox/Tc-inducible retroviral vectors could not be used successfully, we resorted to the original form of Dox/Tc system in which the gene coding for the tTA or the rTA was on one plasmid and the gene whose expression needed to be regulated on a separate plasmid. As reported previously, we had succeeded in isolating founder clones from both DU-145 and PC-3 cells in which the expression of a reporter plasmid bearing TRE could be induced by either adding or removing Dox/Tc from the medium (Table 2, page 7, previous report). We also obtained founder clones from androgen-responsive LNCaP cells in which the expression of a reporter bearing TRE could be induced by either adding or removing Dox/Tc into the medium from Dr. Kerry Burnstein, University of Miami School of Medicine, Miami, Florida.

The goal of task 2 was to set up a functional Dox/Tc-inducible system and create double-stable Dox/Tc-inducible cell clones derived from PC-3, DU-145 and LNCaP founder clones. To achieve task 2, we engineered a variety of Cx32 and Cx43 cDNAs, subcloned them into plasmid pTRE2, and plasmid pBI in which the expression of 2 genes can be regulated simultaneously, and optimized conditions for their inducible expression upon removal of Dox/Tc. The results of these experiments are shown in **Figure 1** (page 10). These results are summarized as follows:

1. The best plasmid concentration for the induction of various genes cloned into pTRE2 and pBI was found to be 0.625 μg/well in a six well cluster (*Figure 1 B, page 10*).

2. The expression of Cx genes can be visualized microscopically, suggesting that the optimized conditions can be used to study the assembly of Cxs into gap junctions without resorting to more complicated biochemical assays (*Figure 1 C, page 10*).

Task 3

The aim of task 3 was to determine whether treatment with RAR- and RXR-specific analogs of vitamin A will induce the formation of gap junctions in LNCaP and PC-3 clones expressing Cx32 and Cx43 constitutively or inducibly as observed with all-trans-13-retinoic acid (ATRA), an analog of vitamin A that activates both RARs and RXRs.

Various natural and synthetic analogs of retinoids, obtained from Biomol Research Laboratories (Plymouth Meeting, PA), are shown in the table (*page 6*). The results of all these experiments are described below. The salient features of our data, also described in part in previous report, have been verified and further substantiated by quantitative immunocytochemical, western

blot and functional analysis as summarized in *Figures 2, 3 and 4 (pages 10-14):*

(1) ATRA, 13-cis-retinoic acid and 4-hydroxyphenretinamide (4-HPR) did not induce the formation of gap junctions in Cx-lacking wild-type LNCaP or PC-3 cells (data not shown).

(2) At non-toxic concentrations, retinoids enhanced the formation of gap junctions in Cx32 and Cx43-expressing LNCaP cells (*Figures 2 and 3, pages 11, 12*).

(3) Compared to 4-HPR and ATRA, 9-cis-retinoic acid inhibited the formation of gap junctions composed of both Cx32 and Cx43 as judged by triton-X-100 (TX-100)-insolubility assay at 100 nm (*Figure 3 A, page 11*) but had opposite effects had higher concentration (*Figure 2 C, page 11*).

(4) 4-HPR did not facilitate the formation of gap junctions in Cx43- and Cx32-expressing androgen-independent PC-3 cells (data not shown).

(5) We have found that the effects of 9-Cis-retinoic acid on the formation of gap junction are complex and concentration dependent in Cx32 an Cx43-expressing LNCaP cells.

The Specificity Retinoids and the Concentrations Used			
Specific Analog of Retinoid used	*Receptor Specificity*	*Nontoxic Concentration tested*	*Effect on formation of Gap Junctions**
All-trans-retinoic acid	RAR Agonist	10^{-7} M	10
13-Cis- Retinoic acid	RAR and RXR	10^{-6} M	8
9-Cis-Retinoic acid	RXR Agonist	10^{-7} M	Not investigated
4-Hydroxyphenylretinamide	RAR and RXR	10^{-7} M	5
AM-580	RAR Agonist	10^{-6} M	Not investigated
TTNPB (Ro13-7410)	RAR Agonist	10^{-6} M	Not investigated

Task 4

The aim of this task was to determine whether 4-HPR, a potent antineoplastic analog of vitamin A of unknown ligand specificity, is more effective in inducing the formation of gap junctions in LNCaP and PC-3 clones expressing Cx32 and Cx43 constitutively or inducibly, and ascertain whether its effects are consistent with its potent chemopreventing activity.

The effect of 4-HPR on the formation of gap junctions was studied by TX-100 insolubility assay and compared with ATRA and 9-cis-retinoic acid. The results are summarized below and shown on page 12 (*Figure 3 A*).

The data document that 4-HPR seems to be as potent as ATRA in inducing the assembly of gap junctions composed of Cx32 as judged by TX-100-insolubility assay (*Figure 3 A*) but does not seem to be more potent in inducing the assembly of connexins into gap junctions as judged by immunocytochemical assays (data not shown).

Hence, the hypothesis that the more potent anti-neoplastic effects of 4-HPR are due to its ability to modulate the assembly of Cxs into gap junctions was not supported by these data. It remains to be seen whether the effect of 4-HPR on the formation of other class of cell junctions, such as adherens junctions and tight junctions, correlate with its more potent ani-neoplastic effects.

Task 5

The aim of task 5 was to ascertain whether EB-1089, a less hypercalcemic but more potent analog of 1,25 dihydroxyvitamin D (1,25 D), is more effective in inducing the formation of gap junctions in LNCaP and PC-3 clones expressing Cx32 and Cx43 constitutively or inducibly. *We have not as yet studied the effect of EB-1089 alone* on the formation of gap junctions and plan to initiate these studies in future.

Task 6

The aim of task 6 was to determine, using different concentrations and durations of treatment with vitamin A and D analogs, the most potent and the least toxic treatment regimes that are effective in inducing the formation of gap junctions maximally in LNCaP and PC-3 clones expressing Cx32 and Cx43 constitutively or inducibly.

In the first series of experiments we studied the time course of formation of gap junctions in LNCaP cells expressing Cx43 and Cx32 using ATRA (*Figure 2 B, page 11*). The solubility of Cxs in Triton X100 was used as an assay to study the formation of gap junctions biochemically (*see Figure 2 B legend, page 11*). The results show that enhancement of formation of gap junctions induced by ATRA is slow and manifests maximally at 48 h post-treatment. Moreover, we also studied the formation of gap junctions by functional assays, by injecting gap junction permeable fluorescent tracer lucifer yellow in control cells and cells treated with ATRA and 9-cis-retinoic acid (*Figure 2 C, page 11*). Our results show the following:

1. Both ATRA and 9-cis-retinoic acid are equipotent in inducing the assembly of gap junctions as judged by biochemical and functional assays.

2. Higher concentrations of both retinoids inhibited the formation of gap junctions as judged by functional assays.

Task 7

The aim of task 7 was to determine if there is additivity and/or synergism among various analogs of vitamin A and D in inducing the formation of gap junctions in LNCaP and PC-3 clones expressing Cx32 and Cx43 constitutively or inducibly.

We have found that there is no additivity between retinoids and vitamin D in enhancing and inhibiting the formation of gap junctions composed of Cx32 and Cx43 in androgen-responsive LNCaP and androgen-independent PC-3 cells that express these Cxs(data not shown).

Tasks 8 and 9

The aim of tasks 8 and 9 was to investigate whether the formation of gap junctions composed of Cx32 and Cx43 is regulated differently by various analogs of vitamin A and D in androgen-dependent (LNCaP) and -independent (PC-3) clones expressing these Cxs constitutively or inducibly.

We have found that the retinoids and various analogs of vitamin D do not modulate the formation of gap junctions composed of connexin32 and connexin43 in androgen-independent PC-3 cells in which both Cxs accumulate intracellularly and do not reach the plasma membranes (see appended manuscript). We have also found that both retinoids and vitamin D3 enhance the formation of gap junctions composed of Cx32 and Cx43 in LNCaP cells (*Figure 3 B, page 12*).

Tasks 10, 11 and 12

The aim of these tasks was to investigate if the assembly of E-cadherin and ZO-1— the 2 putative tumor suppressor genes— into cell junctions is augmented by treatment with 4-HPR, RAR- and RXR-specific analogs of vitamin A,1,25 D, and EB-1089 only in LNCaP and PC-3 clones expressing Cx32 and Cx43 constitutively or inducibly but not in Cx-lacking wild-type LNCaP cells.

We have found that the assembly of E-cadherin and ZO-1 into cell junctions is enhanced by both retinoids and vitamin D3. The results of these experiments are shown in Figure 3 B (*page 12*) and Figure 4 (*page 13*) for other cell junction associated proteins.

3. Key Research Accomplishments

- Isolation of founder clones from androgen-responsive LNCaP and androgen-independent PC-3 cells and the demonstration of the inducibility of the expression of Cxs as well as other tumor suppressor genes upon addition of Dox/Tc.

- Demonstration that the retinoid and vitamin D3-induced enhancement of the assembly of Cx32 and Cx43 into gap junctions in LNCaP cells augments the recruitment as well assembly of E-cadherin and ZO-1 to the areas of cell-cell contact.

- Demonstration of the inability of various natural and synthetic analogs of vitamin A and vitamin D3 to recruit and enhance the assembly of E-cadherin and ZO-1 at the areas of cell-cell contact in LNCaP and PC-3 cells.

- Demonstration that the retinoids and vitamin D3 do not affect the assembly as well as trafficking of both Cx32 and Cx43 in androgen-independent PC-3 cells in which these Cxs accumulate intracellularly.

4. Reportable Outcomes

- The key research accomplishments supported by this award were reported at the 40th American Society for Cell Biology Annual Meeting December 9-13, 2000, San Francisco (see appendix).

- A paper showing alterations in cell adhesion molecule and in Cx43 and Cx32 expression in rat prostate model, which is partly supported by this award, has been published (see appendix).

- The key research accomplishments supported by this award and reported briefly in this report were presented at the "2001 International Gap Junction Conference" Honolulu, Hawaii, August 4-9(see appendix).

- The key research accomplishments supported by this award and reported briefly in this report were presented at the special American Association for Cancer Research proceedings entitled" New Discoveries in Prostate Cancer Biology and Treatment " held on December5-9, 2001, The Registry Resort Naples, Florida (see appendix).

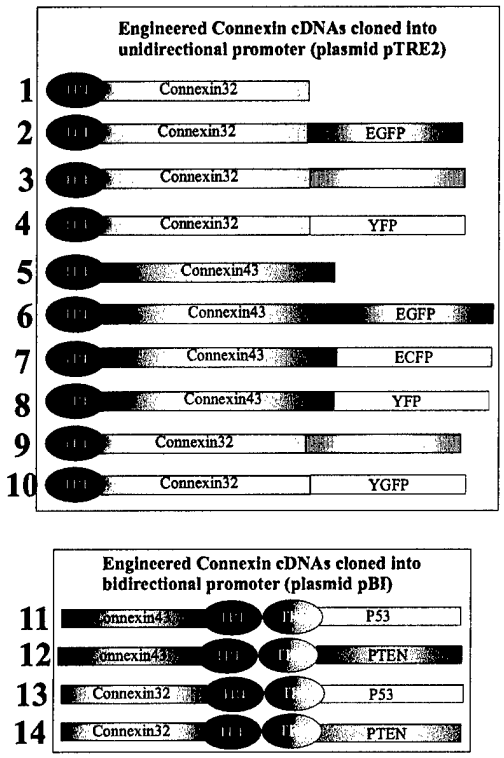
- A manuscript showing intracellular accumulation of Cx43 and Cx32 in androgen-independent PC-3 cells and its mitigation by α-catenin, supported by this award, has been prepared for submission for the publication (see appendix).

5. Conclusions

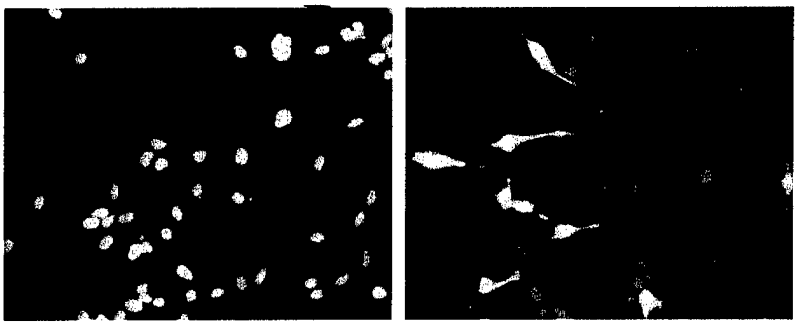
We have hypothesized that chemopreventive agents — such as the natural and synthetic analogs of vitamin A and D — inhibit growth and/or induce differentiation of prostate cancer cells by regulating the assembly of connexins into gap junctions which in turn facilitates the assembly of other cell adhesion molecules and their associated proteins, such as E-cadherin and catenins, into

FIGURE 1

A

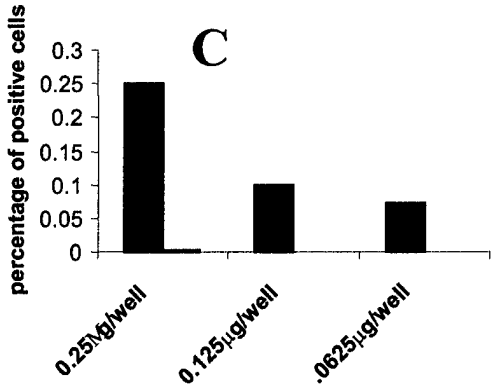


C.The constructs shown above were engineered using standard recombinant DNA protocols. Constructs 1 and 5 are the standard wild-type Cx32 and Cx43 cloned into plasmid pTRE2 in which the expression of these genes can be induced upon removal of Doxicycline (Dox). Constructs 2-4 and 6-9 are chimeras of Cxs fused to various enhanced fluorescent proteins and cloned into pTRE2. Constructs 10-14 contain both Cxss and the well-known tumor suppressor genes (p53 and PTEN) cloned into bidirectional promoter pBI in which the expression of both genes is induced upon removal of



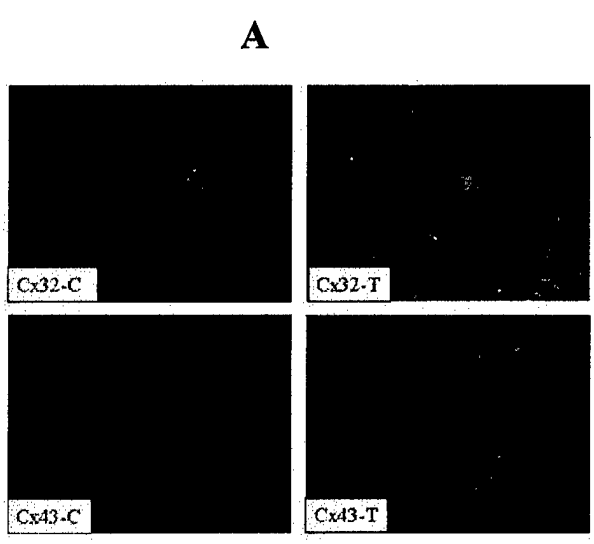
DOX NO DOX

B.1x10^5 cells of Tc-inducible LNcaP clone were seeded in 6-well clusters and grown for 24 h in 2ml medium. Transient transfection of pTRE2rCx32 (0.25μg/well) with FuGENE 6 was carried out at the ratio of plasmid DNA/FuGENE of 1:4 (μg/μl). In control wells Dox at 2μg/ml Dox was added to suppress the induction. Immunochemical staining for Cx32 was performed 48 h post-transfection and detected with Alexa-594 (red) conjugated 2nd antibody. After washing, the cells were counterstained with DAPI (blue), and imaged with Openlab 3 imaging program. Note the induction of Cx32, as judged by the appearance of red cells, upon

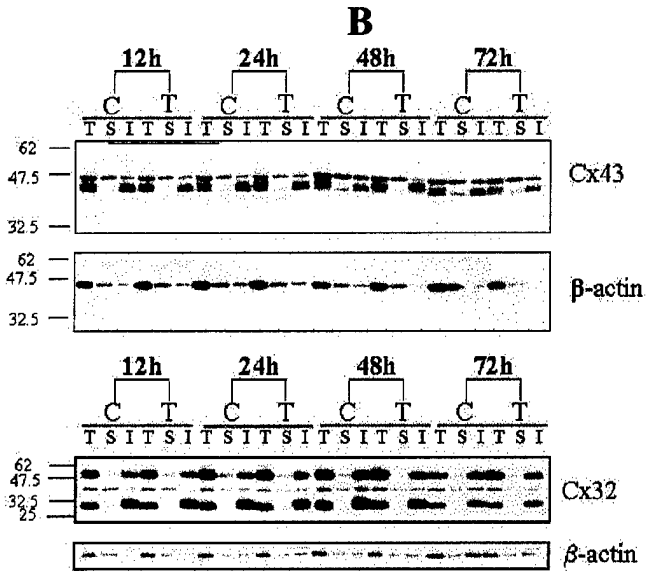


C.1x10^5 cells of Tc-inducible LNcaP clone were seeded in 6-well clusters and grown for 24 h in 2ml medium. Cells were then transiently transfected with of pTRE2rCx32-YFP at the indicated concentrations B with FuGENE 6 was carried out at the ratio of plasmid DNA/FuGENE of 1:4 (μg/μl). In control wells Dox at 2μg/ml Dox was added to suppress the induction. Induction of the reporter gene was assessed by counting the number of fluorescent cells per micrsocopic field at 48 h post-transfection. Note that the best induction is achieved at the DNA concentration of 0.250μg/well and that there is no basal level of expression. When the concentration of DNA was increased to 2μg/well, there was a

Figure 2

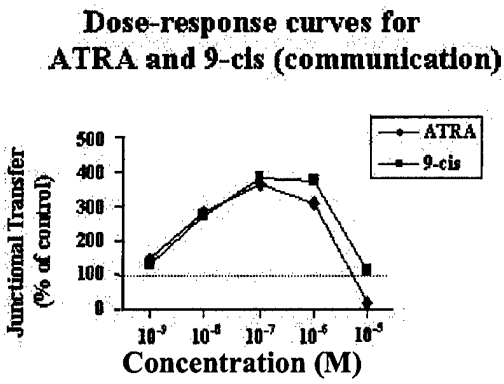


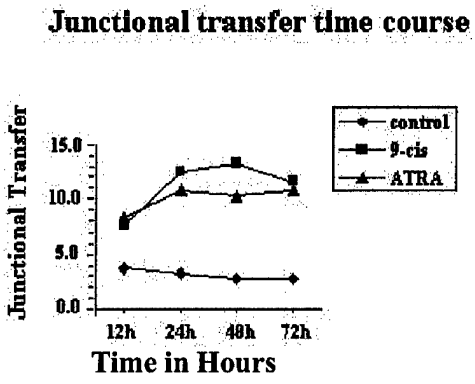
A. Cx43 and Cx32-expressing LNCaP cells were treated with 9 Cis-retinoic acid for 24 h and immunostained (green) with Cx-specific antibodies. The nuclei (red) were stained with DAPI. Note that 9- Cis retinoic acid treatment



B. Total, (T), Triton X 100-soluble (S) and insoluble (I) extracts from LNCaP expressing Cx43 and Cx32 were analyzed by western blot analysis at various times after treatment with ATRA at 100 nM, a concentration which is not toxic to these cells. Note that maximum effect on insoluble fraction (formation of gap junctons) manifests only at 48 h

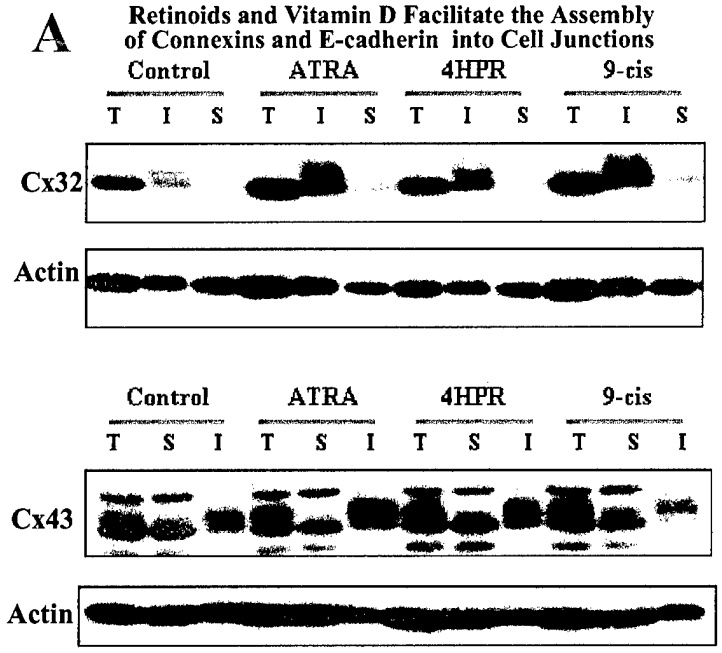
C



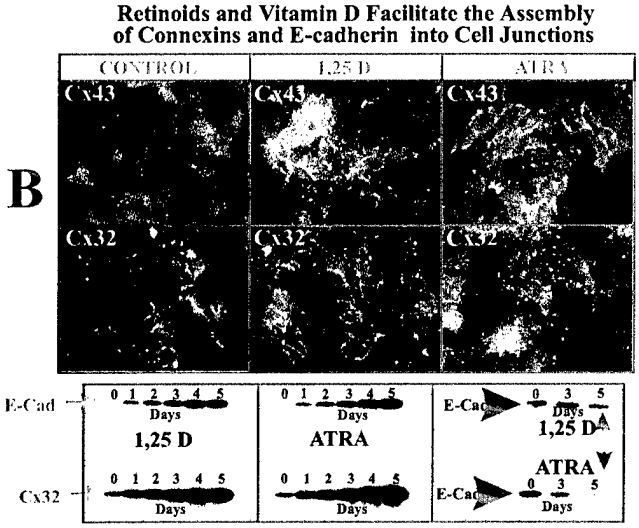


C. Right Figure:50,000 connexin43-expressing LNCaP cells were seeded in 6 cm plastic dishes and allowed to grow for 24 h. Cells were then treated with various retinoids at 100 nm and the junctional transfer of lucifer yellow was assesses as described in our previous studies ant the attached submitted manuscript. Note that consistent with the immunocytochemical and biochemical data, enhancement of junctional communication is slow and begins to be observed at 12 h post-treatment and maximum effect is observed at 48 h post-treatment Left Figure: 50,000 connexin43-expressing LNCaP cells were seeded in 6 cm plastic dishes and allowed to grow for 48 h. Cells were then treated with various doses of retinoids for 48 h and the junctional transfer of lucifer yellow was observed by microinjection as described previously. Note that the most opotimal cocentration of variou retinoids in enhancing communication

Figure 3



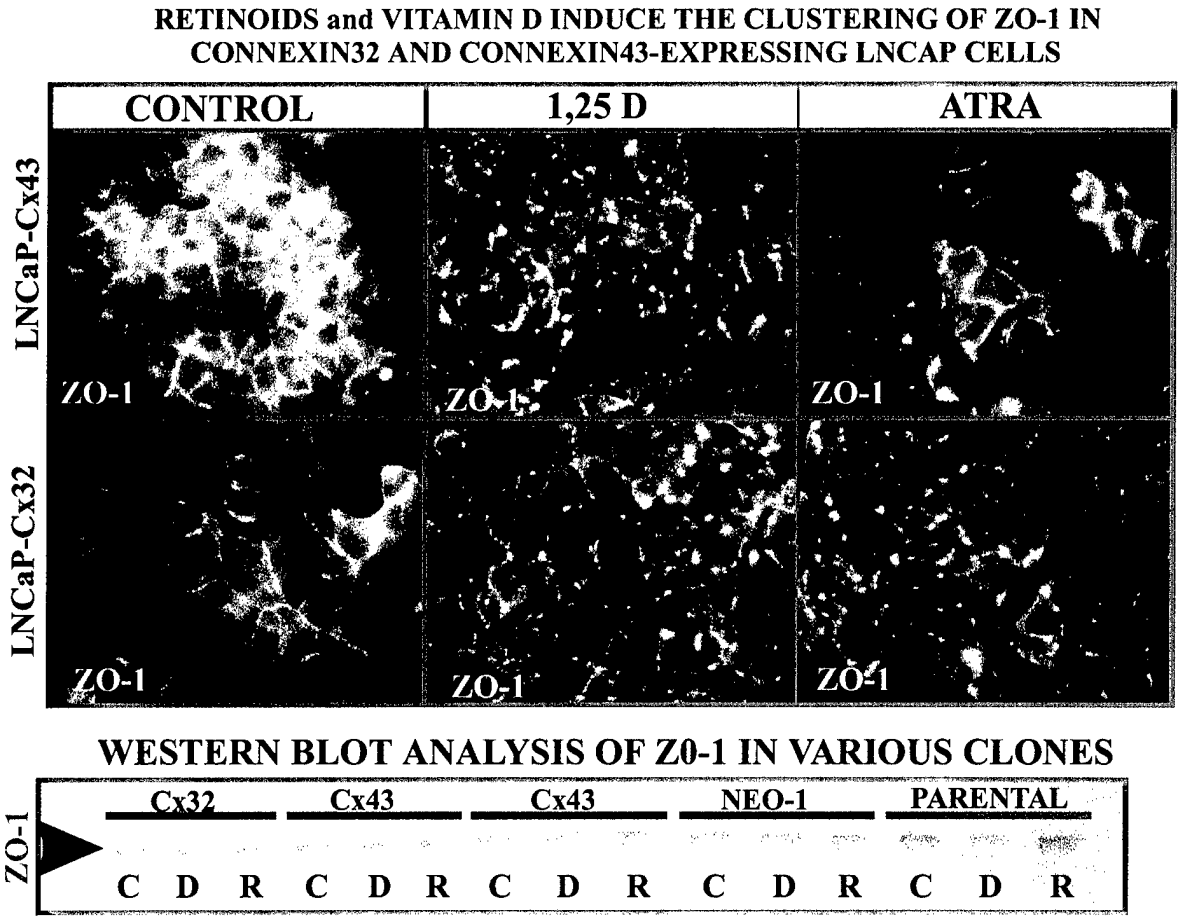
A. Retinoids enhance the assembly of Cx43 into gap junctions as judged by Triton X-100 solubility assay. Cx43 and Cx32-expressing LNCaP cells were seeded in 10 cm dishes and allowed to grow to 70 % confluence. Cells were then treated with various retinoids at 100 nm and the assembly of Cx43 and Cx32 was assessed biochemical by Triton-X-100 insolublility assay as described in the appended submitted manuscript. Note that all retinoids enhanced the triton-X-100-



B. Top: Vitamin D3 (1,25 D) is as potent in enhancing the assembly of Cx43 and Cx32 into gap junctions as various classes of retinoids. 50, 000 cells were seeded on glass coverslips and allowed to grow to 70 % confluence. Cells were then treated with 10 nm 1,25 D and 100 nm ATRA and the formation of gap junctions was studied with immunocytochemistry using Cx-

Bottom: Treatment withVitamin D and All-trans retinoic acid Facilitates the Assembly of Cx32 and E-cad into Cell Junctions. 500,000 cells from Cx32-expressing (Cx-32B) and Cx-lacking (NEO) clones of LNCaP cells were seeded in 10 cm dishes and treated with 1,25D (10 nM) and ATRA (100 nM) for various days. Assembly of Cx32 into GJs and that of E-cad into CJs was analyzed by western blots using TX-100 insolubility assay (1 % for Cx32 and 0.5 % for E-cad) at various days after treatment as described in the appended submitted manuscript. Under these conditions, E-cad and Cx32 which are not assembled into CJs are solubilized, whereas CJ-associated E-cad and Cx32 remain insoluble. Note that treatment with 1,25 D and ATRA increased the assembly of E-cad and Cx32 intoCJs in Cx-expressing

FIGURE 4



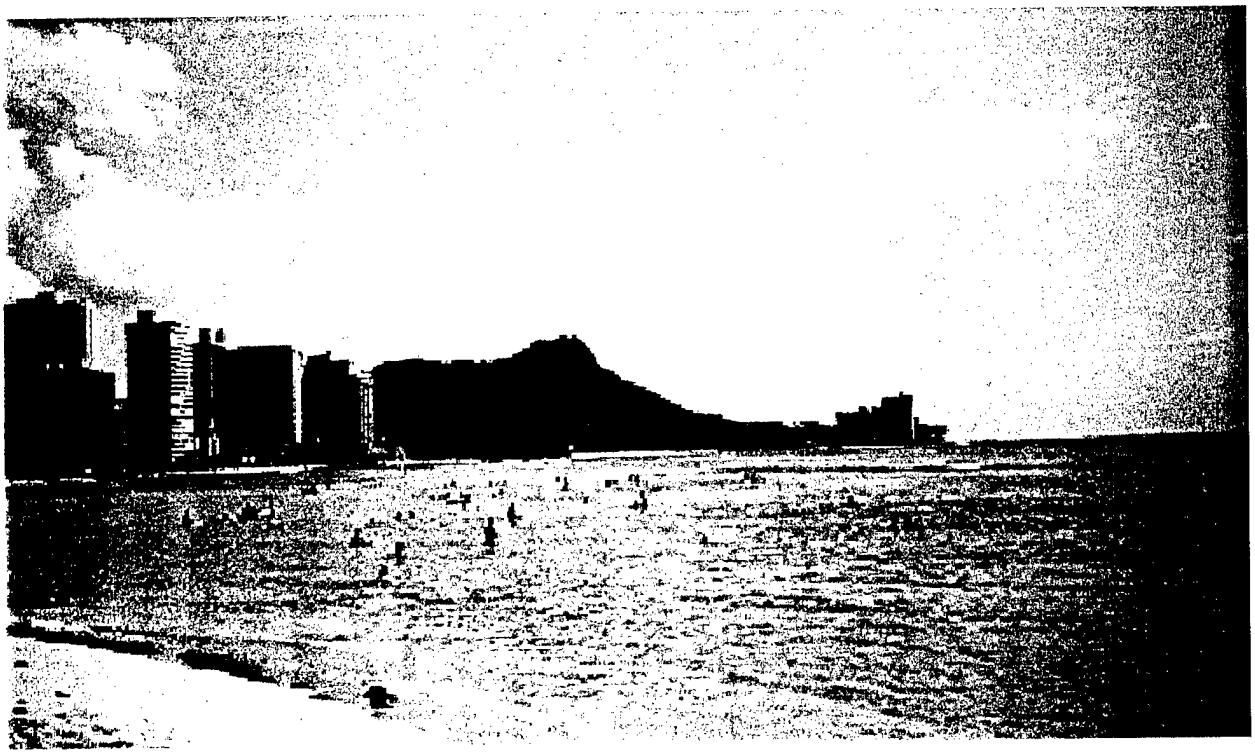
Top:Treatment withVitamin D and All-trans retinoic acid Facilitates the Assembly of Cx32 and ZO-1 into Cell Junctions. 50,000 cells, from a LNCaP clone cells expressing Cx32 and C43 were seeded on glass coverslips. After 24 h, cells were either left untreated (controls) or treated with vitamin D3(1,25D, 10 nM) and all-trans-retinoic acid(ATRA, 1 μM) for 48 h. Recruitment of ZO-1 at the cell-cell contact areas was studied by immunocytochemical analaysis. Note that in untreated cells the pattern of ZO-1 staining is not punctate and that treatment with 1,25 D and ATRA enhanced the localization of ZO-1 at the areas of cell-cell contact. Bottom: To rule out the possibility that increased staining at the cell-cell contact area was due to increased sythesis of ZO-1, 1,25 D and ATRA treated cells were analyzed by western blot analysis. Note that 1,25 D and ATRA do not alter the expression of ZO-1 but only affect its localization..

cell junctions. We had further proposed that chemoprevention of PCA by retinoids and 1,25 D would ideally proceed if methods to assess their impact on premalignant and malignant lesions could be identified and strategies designed to direct them to those lesions which have the potential to develop into metastatic PCA. Thus our proposed studies were designed to seek answer to the following question: Will investigations focused on the assembly of connexins into gap junctions as intermediate endpoint biomarkers serve as useful guideposts in screening those analogs of retinoids and 1,25 D likely to be most effective in chemopreventing PCA? The data so far we have obtained clearly demonstrates the feasibility of our approach, although much remains to be done and a lot of time was lost during PI's transit from the University of Miami School Of Medicine to the University of Nebraska Medical Center.

6. References

1. Mehta, P.P., Bertram, J.S. and Loewenstein, W.R. 1986. Growth inhibition of transformed cells correlates with their junctional communication with normal cells. Cell: 44: 187-196.
2. Mehta, P.P., Bertram, J.S., and Loewenstein, W.R. 1989. The actions of retinoids on cellular growth correlate with their actions on gap junctional communication. J. Cell Biol. 108: 1053-1065.
3. Mehta, P.P. and Loewenstein, W.R. 1991. Differential regulation of communication by retinoic acid in homologous and heterologous junctions between normal and transformed cells. J. Cell Biol. 113: 371-379.
4. Mehta, P.P., Hotz-Wagenblatt, A., Rose, B., Shalloway, D. and Loewenstein, W.R. 1991. Incorporation of the gene for a cell-cell channel protein into transformed cells leads to normalization of growth. J. Membr. Biol. 124:
5. Mehta, P.P., Lokeshwar, B.L., Schiller, P.C., Bendix, M. V., Ostenson, R.C., Howard, G. A., and Roos, B. A. 1996. Gap-junctional communication in normal and neoplastic prostate epithelial cells and its regulation by cAMP. Mol. Carcinogenesis, 15, 18-32.
6. Mehta, P. P., Perez-Stable, C., Nadji, M., Mian, M., Asotra, K., and Roos, B.A. (1999). Suppression of human prostate cancer cell growth by forced expression of connexin genes. Dev. Genet. 24:91-110
7. Mehta, P.P., Zhao, S., and Song, X-H (2000). Overexpression of connexin43 in adnrogen-independent prostate cancer cell lines results in intracellular accumulation and abnormal phosphorylation. Mol Biol Cell 11: 225a.
8. Habermann, H., Chang, W.Y. Birch, L., Mehta, P.P., and Prins, G.S. (2001). Developmental exposure to estrogens alters epithelial cell adhesion and gap junction proteins in the adult rat prostate. Endocrinology: 142:359-369.
9. Govindrajan, R., Guo, R-J., and Mehta, P.P. (2001). Defective trafficking of connexin43 and connexin32 in an androgen-independent human prostate cancer cell line, PC3. 2001 International Gap Junction Conference, Hawaii, August 4-9.
10. Mehta, PP., Xiao F., and Govindarajan R (2001). Retinoids and vitamin D3 modulate junctional communication and the distribution of connexin32 and connexin43 containing gap junctions in human prostate cancer cells. American Association for Cancer Research "New Discoveries in Prostate Cancer Biology and Treatment" Special Meeting, December 5-9, Naples, Florida.
11. Govindarajan, R., Zhao, S., Wheelock, MR., Johnson, KR., and Mehta, PP (2002). Impaired trafficking of connexins in human androgen-independent cell lines and its mitigation by α-catenin. Manuscript prepared for submission. (A

2001 International Gap Junction Conference



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Retinoids and vitamin D3 modulate junctional communication and the distribution of connexin32 and connexin43 containing gap junctions in human prostate cancer cells
Parmender P. Mehta, Xiao-Hong Song, and Feng Xiao

Besides cell-cell and cell-matrix adhesion cell junction(CJ)s, epithelial cells form a specialized class of CJs called gap junction(GJ)s through which small molecules can be exchanged between cells. The GJs are bicellular structures formed by the family of 20 related proteins named connexin(Cx)s, which assemble into hexamers to form connexons that join with connexons in adjacent cells to form intercellular channels. Gap junctional communication (GJC) has been proposed to play a role in the maintenance of tissue homeostasis and the initiation of neoplasia. Our previous studies have shown that, compared to normal prostate epithelial cells, GJC in prostate cancer (PCA) cell lines was either absent or reduced (Mehta et al. Mol Carcinog 15: 18-32, 1996). Our studies also showed that the forced expression of Cx32 and Cx43— the 2 Cxs expressed by the well-differentiated epithelial cells of the prostate — into an indolent, androgen-responsive and Cx-deficient PCA cell line, LNCaP, induced the formation of GJs, enhanced GJC, and inhibited growth(Mehta et al. Dev Genet 24: 91-110, 1999). Although it is well-established that the chemopreventive effects of the retinoids and vitamin D3 are related to their ability to influence the state of differentiation, proliferation, and apoptotic death of tumor cells, the molecular mechanisms responsible for these effects remain still equivocal as most genes regulated by them are as yet unknown. Using clones of LNCaP cells that over express Cx43 and Cx32, we have examined and compared the effect of vitamin D3 and RAR- and -RXR-selective retinoids on GJC and the formation and distribution of GJs. Our results show that treatment of Cx32- and Cx43-expressing LNCaP cells with the retinoids and vitamin D3 enhanced GJC further by 2-3 folds as judged by microinjecting 443 Da fluorescent tracer lucifer yellow. Enhancement of GJC was observed as early as 8 h after treatment and continued to be observed until 72 h. Enhancement of GJC did not result from an increase in the synthesis and/or assembly of Cx43 and Cx32 into GJs but was accompanied by the redistribution of GJs at the cell-cell contact areas. Moreover, our results also show that treatment with the retinoids and vitamin D3 facilitated the recruitment of ZO-1 (a tight junction- associated protein), to the cell surface in Cx32- and Cx43-expressing LNCaP cells. These effects were not observed in Cx-lacking parental LNCaP cells. Furthermore, we also investigated whether treatment with the retinoids and vitamin D3 would render Cx43 and Cx32-expressing LNCaP cells more susceptible to the growth-modulatory effects of these agents. Our data show that the retinoids and vitamin D3 treatment altered the morphology of Cx32- and Cx43-expressing LNCaP cells profoundly whereas no significant effect was observed in Cx-lacking parental LNCaP cells. Our data support the notion that the chemopreventive, growth inhibitory and differentiation- and apoptosis-inducing effects of retinoids and vitamin D3 in prostate epithelial cells may result from their ability to control the formation and dissolution of GJs that in turn modulates the formation of other CJs and their associated proteins.

0013-7227/01/$03.00/0
Endocrinology

Vol. 142, No. 1
Printed in U.S.A.

Developmental Exposure to Estrogens Alters Epithelial Cell Adhesion and Gap Junction Proteins in the Adult Rat Prostate*

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ABSTRACT

Brief exposure to estrogens during the neonatal period interrupts rat prostatic development by reducing branching morphogenesis and by blocking epithelial cells from entering a normal differentiation pathway. Upon aging, ventral prostates exhibit extensive hyperplasia and dysplasia suggesting that neonatal estrogens may predispose the prostate gland to preneoplastic lesions. To determine whether these prostatic lesions may be manifested through aberrant cell-to-cell communications, the present study examined specific gap junction proteins, Connexins (Cx) 32, and Cx 43, and the cell adhesion molecule, E-cadherin, in the developing, adult and aged rat prostate gland. Male rat pups were given 25 μg estradiol benzoate or oil on days 1, 3, and 5 of life. Prostates were removed on days 1, 4, 5, 6, 10, 15, 30, or 90 or at 16 months, and frozen sections were immunostained for E-cadherin, Cx 43, and Cx 32. Colocalization studies were performed with immunofluorescence using specific antibodies for cell markers. Gap junctions in undifferentiated epithelial cells at days 1–10 of life were composed of Cx 43, which always colocalized with basal cell cytokeratins (CK 5/15). Cx 32 expression was first observed between days 10–15 and colocalized to differentiated luminal cells (CK 8/18). Cx 43 and Cx 32 never colocalized to the same cell indicating that gap junction intercellular communication differs between basal and luminal prostatic cells. While epithelial connexin expression was not initially altered in the developing prostates following estrogen exposure, adult prostates of neonatally estrogenized rats exhibited a marked decrease in Cx 32 staining and an increased proportion of Cx 43 expressing cells. In the developing prostate, E-cadherin was localized to lateral surfaces of undifferentiated epithelial cells and staining intensity increased as the cells differentiated into luminal cells. By day 30, estrogenized prostates had small foci of epithelial cells that did not immunostain for E-cadherins. In the adult and aged prostates of estrogenized rats, larger foci with differentiation defects and dysplasia were associated with a decrease or loss in E-cadherin staining. The present findings suggest that estrogen-induced changes in the expression of E-cadherin, Cx32 and Cx43 may result in impaired cell-cell adhesion and defective cell-cell communication and may be one of the key mechanisms through which changes toward a dysplastic state are mediated. These findings are significant in light of the data on human prostate cancers where carcinogenesis and progression are associated with loss of E-cadherin and a switch from Cx32 to Cx43 expression in the epithelium. (*Endocrinology* **142:** 359–369, 2001)

BRIEF exposure of male rats to estrogens during the neonatal period has been shown to retard prostate growth, branching morphogenesis and epithelial differentiation during development, and to permanently alter its secretory function and response to androgens during adulthood (1–4). This process, referred to as neonatal imprinting or developmental estrogenization, is associated with an increased incidence of prostatic intraepithelial neoplasia and hyperplasia with aging (3, 5, 6). Thus, neonatal estrogenization of the rat has evolved as a useful model for evaluating the role of exogenous and endogenous estrogens as a predisposing factor for prostatic diseases later in life (7). The mechanisms of this estrogenic effect on the prostate are not fully understood. Estrogen action in the rat prostate gland is mediated through estrogen receptor α (ERα) and estrogen receptor β (ERβ). In the normal developing prostate, ERα expression is confined to periductal mesenchymal cells surrounding the proximal ducts, whereas ERβ is expressed at low levels in the undifferentiated epithelial cells (8, 9). Although neonatal exposure to estrogens does not initially alter ERβ expression (9), there is an immediate up-regulation of ERα expression within periductal stromal cells along the length of the developing ducts that allows for amplification of estrogenic signals in these stromal cells at the time of neonatal exposure (8). We have previously shown that neonatal estrogen exposure initially blocks prostatic epithelial cells from entering a normal differentiation pathway as characterized by a lack of $p21^{cip\text{-}1/waf\text{-}1}$, a delay in the appearance of luminal cell markers, a retention of a continuous layer of basal cells along the basement membrane, and loss of androgen receptor (AR) (10, 11). Differentiation defects of the epithelium can be permanent as evidenced by reduced adult expression of epithelial ERβ and decreased expression of several secretory proteins including prostate binding protein (PBP), urokinase and a 21-kDa secretory protein (4, 9, 10, 12). In addition, we have recently shown that neonatal estrogens stimulate the proliferation of a zone of fibroblasts beneath the basement membrane that may impede ductal branching and cell-cell interactions between smooth muscle and epithelial cells (12).

Received July 11, 2000.
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* Supported by NIDDK Grants 40890 and 09873, NCI CA-73769, DOD (DAMD17-00-1-0032), VA Merit Review Award, and a travel scholarship from AESCA GmbH. Traiskirchen, Austria and AstraZeneca GmbH, Vienna, Austria.

Cell junctions, particularly abundant in epithelial cells, are macromolecular structures that are essential for intercellular adhesion and communication. Signal transduction through cell junction-associated adhesion molecules has been implicated in the control of proliferation and differentiation of epithelial cells during normal and neoplastic development (13). The membrane channels of gap junction (GJ)s, formed of proteins called connexin(Cx)s, provide an intercellular pathway for the diffusion of small growth-regulatory molecules (≤1 kD) between the cytoplasmic interiors of contigous cells (14), a process commonly referred to as gap junctional intercellular communication (GJIC) (15, 16). The presence of GJs in most tissues at early embryonic development and the recent demonstration of mutations in Cx genes in certain human genetic disorders point to crucial biological roles of GJIC (14). The cell-to-cell channels are bicellular structures formed by members of the Cx family of related proteins that first are assembled into hexamers to form connexons that align and join with connexons in adjacent cells to form intercellular pathways (17). Currently, 16 distinct Cx genes, which are designated according to their molecular mass, have been cloned (18). Although all Cxs share a similar topology, some Cxs are expressed in a tissue-specific manner, form channels of different permeabilities, and show specificity in docking to other Cxs (14). While GJIC has been long postulated to fulfill the roles of intercellular buffering of cytoplasmic ions and synchronization of cellular behavior, direct evidence for these roles has only recently been obtained from gene knockout studies (14). In addition to well-documented role of GJIC in the maintenance of tissue homeostasis and cellular behavior, evidence is mounting that bidirectional signaling between cell adhesion molecules, such as E-cadherin, and Cxs may be crucial for controlling embryonic development (19, 20).

The ability of cells to form GJs with some cells and not with others enables the formation of "communication compartments" that may allow the restriction of morphogens to appropriate developmental regions (15, 21). Because GJIC has been shown to be modulated by a variety of agents—such as growth factors, hormones, and oncogenes—it is possible that these agents affect cell growth, differentiation, and tissue homeostasis by modulating the expression and function of Cxs. The spatio-temporal expression of several Cx genes during embryonic development and organogenesis is well established (21, 22). In the limb bud, for example, GJs are found between epithelial cells as well as between mesenchymal cells where they are distributed in the form of a gradient (23). Presently, there are no reports on connexin expression and patterning in the developing prostate gland. In the adult rat prostate, as in other exocrine glands, Cx32 and Cx26 have been found on secretory epithelial cells, whereas Cx43 was not observed (24). Reports on connexin expression in normal adult human prostate are controversial. While some investigators found Cx32 and no Cx43 expression (25, 26), others reported the presence of Cx43 in benign prostate cells (27). It is significant to note that GJ-protein expression is regulated, in part, by steroid hormones in steroid sensitive organs. Importantly, Cx43 messenger RNA levels are up-regulated by estrogens in myometrial cells (28). Labor and estrogens increase Cx43 in rat myometrium while progesterone suppresses Cx43 expression (29) via increased c-*fos* messenger RNA (30). Additionally, testosterone has been shown to increase Cx32 expression in spinal cord motorneurons (16).

For GJ to form, the members of the interacting cells must be close to one another and cell adhesion molecules (CAMs) seem to be involved in this coupling process (31) and in regulating connexin expression (19, 20, 32). Vice versa, the formation of adherens junctions is impaired if GJIC is blocked (18). CAMs are transmembrane proteins that bind in a homophilic manner and form belt-like cell-cell adhesion. CAMs are grouped into two categories: the cadherins, the cell adhesive properties of which are Ca^{2+}-dependent, and the immunoglobulin superfamily CAMs. Among the cadherins, E(epithelial)-cadherin is expressed first on all early mammalian embryonic cells and is later restricted to epithelial tissues of embryos and adults (33, 34). Cadherins transmit intercellular signals through catenin proteins that connect the cytoplasmic region of the cadherins to the cytoskeleton of the cell. Cadherins are extremely important in establishing and maintaining cell-cell interactions between epithelial cells and in adults, are critical for retaining epithelial integrity (34).

In adult cells, loss of E-cadherin is associated with malignancy (35, 36). Likewise, the majority of neoplastic cells have fewer GJ and GJIC is impaired compared with non-neoplastic cells (16). In prostate carcinoma cells, a decrease in Cx43 expression has been reported compared with benign prostate cells and eventual loss of both connexins 32 and 43 occurs in advanced carcinoma (25, 27). Restoration of connexin gene expression and GJIC *in vitro* and *in vivo* by gene therapy can reduce tumor cell growth (36, 37). Similarly, reconstitution of an epithelial-specific CAM (C-CAM) via gene therapy was found to repress tumor growth in prostate cancer *in vitro* and *in vivo* (38).

Since cell-cell communication through gap junction proteins and E-cadherins are critical for normal development, since their expression can be regulated by steroids and since aberrant expression is associated with dysplasia and tumor formation, we were interested in the present study in determining 1) the ontogeny of connexins and E-cadherin expression in the rat prostate; 2) whether neonatal estrogens could influence their developmental expression; and 3) whether changes in connexin and E-cadherin in estrogenized prostate glands were associated with epithelial hyperplasia and dysplasia in the adult. To accomplish these objectives, developing and adult prostates of control and estrogenized rats were studied by immunocytochemistry using specific antibodies against connexins, E-cadherin and a variety of cell markers in the prostate gland.

Materials and Methods

Animals

All rats were handled in accordance with the principles and procedures of the Guiding Principles for the Care and Use of Animal Research. Timed pregnant female Sprague Dawley rats were purchased from Zivic-Miller Laboratories, Inc. (Pittsburgh, PA) and housed individually in a temperature (21 C) and light (14-h light, 10-h dark) controlled room. Rats were fed Purina rat chow (Ralston Purina Co., St. Louis, MO) *ad libitum*. They were monitored daily for delivery of pups, and the day of

birth was designated as day 0. Pups were sexed according to ano-genital distance, and female pups were removed. All males from a single mother were assigned to one of two treatment groups, given sc injections of either 25 μg estradiol benzoate (Sigma, St. Louis, MO) in 25 μl sesame oil or oil alone on days 1, 3, and 5. Animals were weaned on day 25 and subsequently housed two or three per cage. Pups from both treatment groups were killed by decapitation on days 1, 4, 5, 6, 10, 15, 30, or 90 or at 16 months. Accessory sex gland complexes were quickly removed and placed in ice-cold PBS. Prostatic complexes or individual ventral, dorsal, and lateral lobes were microdissected at 4 C under a dissecting microscope. For immunocytochemistry with frozen sections, tissues were arranged on a nylon square, covered with OCT compound (Miles Laboratories, Elkhart, IN), frozen in liquified propane, and subsequently stored in liquid nitrogen. Tissues used for paraffin sections were fixed in Optiprobe (Oncor, Gaithersburg, MD), gradually dehydrated in alcohol, cleared in xylene and embedded in paraffin.

Immunocytochemistry

Immunocytochemistry was performed according to previously published methods (39). Briefly, frozen prostatic complexes or individual lobes were mounted on precooled chucks (−20 C) in a Reichert-Jung cryostat and sections (6 μm) were thaw-mounted on gelatin-coated glass slides. Whenever possible, individual lobes were sectioned longitudinally to reveal the proximal-distal orientation. At 4 C, the sections were fixed in 2% paraformaldehyde, rinsed, incubated with appropriate 2% blocking serum (goat or horse) and incubated overnight with primary antibody. The specific antibodies, sources and concentrations used are presented in Table 1. As a negative control, normal rabbit (Vector Laboratories, Inc., Burlingame, CA) or mouse IgGs (Zymed Laboratories, Inc., South San Francisco, CA) at 1 μg/ml were substituted for primary antibody on separate sections of all tissues analyzed to determine nonspecific binding. The primary antibody was reacted with biotinylated antigoat or antihorse IgG secondary antibody (Vector Laboratories, Inc.), and detected with an avidin-biotin peroxidase kit (ABC-Elite, Vector Laboratories, Inc.) using diaminobenzidine tetrachloride as a chromagen. The sections were stained with Gill's no. 3 hematoxylin (1:4) as a blue nuclear counterstain. As a final step, the sections were dehydrated gradually with alcohol, cleared with xylene, and coverslipped with Permount (Fisher Scientific, Inc., Itasca, IL).

For immunostaining of the 16-month-old prostate tissues, 6 μm paraffin sections were mounted on silane-coated glass slides (3-aminopropyltriethoxy-silane, Sigma, St. Louis, MO), dried overnight, and baked at 60 C for 30 min, deparaffinized in xylene, gradually hydrated with decreasing concentrations of alcohol, and rinsed in deionized water. Subsequently the slides were heated in a Decloaker pressure chamber (Biocare Medical, Walnut Creek, CA) with DAKO Corp. Target Retrieval solution (DAKO Corp., Carpinteria, CA; 1:10 dilution) for 2 min and rinsed in deionized water for 5 min. Slides were rinsed in PBS and endogenous peroxidase was removed with 250 μl 30% H_2O_2 in 250 ml PBS for 10 min followed by washes in PBS and PBS/gelatin. All other steps were as described for frozen sections.

Photographs of diaminobenzidine tetrachloride stained slides were taken with an Olympus Corp. BHTU microscope system (Olympus Corp., New Hyde Park, NY) using Kodak Ektachrome Elite 100 film (Eastman Kodak Co., Rochester, NY).

Colocalization with fluorescence-immunocytochemistry

The protocol used was similar to that for immunocytochemistry. After overnight incubation with the first primary antibody, the tissue section was incubated with fluorescein-conjugated anti-mouse or anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at 37 C. After washing with PBS/gelatin and incubating with appropriate 2% blocking serum (donkey or goat) at room temperature, sections were incubated with the second primary antibody for 1 h at 37 C (or for actin, overnight at 4 C). Tissues were then incubated with CY3-conjugated anti-rabbit or anti-mouse secondary antibody for 1 h at 37 C and washed with deionized water. Coverslips were mounted with Vectashield containing DAPI (Vector Laboratories, Inc.) to visualize nuclei. Photographs of the fluorochrome-labeled slides were taken using a Carl Zeiss Axioscope (Carl Zeiss, New York, NY) and a Princeton Instruments Microview digital camera (Princeton Instruments, Trenton, NJ). For comparative studies, tissues from different time points as well as from control and estrogenized rats were always run in parallel to reduce discrepancies related to interassay variability in staining intensity. In most instances, photographs comparing treatments and days were taken from tissues processed on the same glass slide. Ventral, dorsal and lateral lobes from 3–6 animals at each time point were evaluated to ensure the reproducibility of results.

Results

1) Ontogeny of connexin 32 and 43 in rat prostate

The results for the ventral, dorsal and lateral lobes were identical throughout these studies, leading us to conclude that lobular differences do not apply to Cx or cadherin expression. At birth, undifferentiated epithelial cells in the developing prostatic ducts expressed Cx43 as discrete punctate staining at the intercellular areas throughout the lengthening ducts (Fig. 1A). In addition, urogenital sinus (UGS) mesenchymal cells also expressed Cx43 abundantly; however, the immunostaining was confined to periurethral mesenchyme and did not extend through the smooth muscle sleeve into the prostatic mesenchyme (Fig. 1, B and C). Over the course of the first 10 days of life, while Cx43 expression in UGS mesenchyme declined to an undetectable level, a gradient of Cx43-specific immunostaining was observed in the developing epithelial ducts whose intensity was the strongest at the distal tips of the buds that had not luminized yet. When the epithelial ducts began to luminize between days 10–15, Cx43 expression was confined to basally positioned cells at the periphery of the ducts, whereas in the luminal cells it was absent (Fig. 1D). The number of Cx43-expressing epithelial cells continued to decline through maturation of the gland. Concomitant with the loss of Cx43 immunostaining in the developing prostate was the appearance of punctate Cx32 immunostaining in epithelial cells along the lateral surfaces of differentiated luminal cells, observed first at day 15 and

TABLE 1. Various antibodies used for immunocytochemistry

Antibody	Animal source	Source	Concentration
Hybridoma supernatant anti-Connexin 32	Mouse	Dr. Parmender P. Mehta (52)	1:2–1:4 (frozen sections) 1:5–1:10 (paraffin sections)
Affinity-purified anti-Connexin 43	Rabbit	Dr. Parmender P. Mehta (53)	1:8000 (frozen sections) 1:4000 (paraffin sections)
Anti-E-Cadherin	Mouse	Transduction Laboratories (Lexington, KY)	0.05 mg/ml
Anti-keratin HMW 903 (for basal cells)	Mouse	Enzo Diagnostics Inc. (Farmingdale, NY)	1:100
Anti-keratin RG 53 (for luminal cells)	Mouse	Cappel/ICN (Aurora, OH)	1:50
Anti-muscle actin HHF 35	Mouse	Enzo Diagnostics Inc. (Farmingdale, NY)	1:50

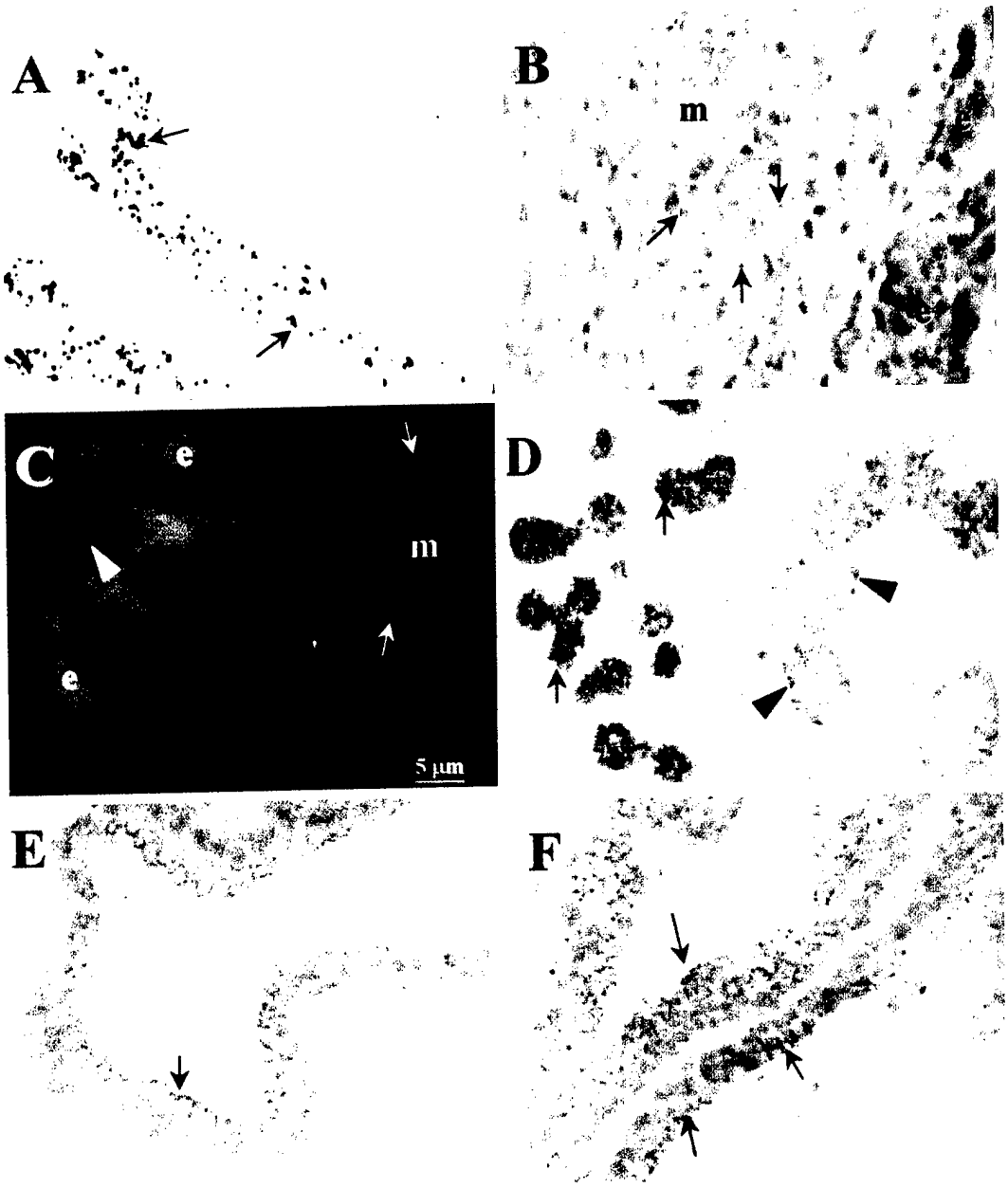

FIG. 1. Connexin 43 and Connexin 32 ontogeny in rat prostate gland. A, Epithelial cells of the day 6 dorsal prostate ducts exhibit punctate Cx 43 stain along undifferentiated epithelial cell borders (*arrows*) throughout the solid cord (×133). B, Day 6 UGS mesenchyme (m) exhibits punctate Cx43 immunostain along mesenchymal cell borders (*arrows*) in addition to the epithelial cells (e) in the proximal ducts (×250). C, Immunofluorescence stain of Cx43 (*red*) in the UGS mesenchyme (*arrows*) and proximal ventral prostate epithelium (*arrowhead*) at day 1 shows discrete punctate staining along all cell borders in this region. D, Day 10 ventral prostate immunostained for Cx43. Solid epithelial cords in the distal prostate exhibit Cx43 along cell borders of all epithelial cells (*arrows*) while ducts that have begun to luminize show Cx43 localization to the basally located epithelial cells (*arrowhead*) (×100). E, Day 30 ventral prostate immunostained for Cx32 shows positive signal along cell borders of epithelial cells (*arrow*) (×250). F, Day 90 ventral prostate epithelium stains strongly for Cx32 in luminal epithelial cells (*arrows*) (×250). All sections are lightly counterstained with hematoxylin for nuclear visualization.

increasing in intensity at day 30 (Fig. 1E) and 90 (Fig. 1F). To characterize further the pattern of expression of Cxs in the basal and luminal cells of rat prostate, we studied the colocalization of Cx43 and Cx32 with each other and with specific cell markers. Cx43 and Cx32 did not localize to the same cells but rather were confined to distinct epithelial cell populations. At day 15, the majority of epithelial cells expressed Cx43, whereas by day 30, the number of cells expressing Cx 43 had declined and there was an apparent increase in the proportion of cells expressing Cx 32. By day 90, the major fraction of prostatic epithelial cells were Cx32 positive, whereas only a minor fraction of cells were Cx43 positive. This shift in Cx43 and Cx32 expressing cells coincided with the known time frame of epithelial cell differentiation in the prostate from undifferentiated basal cells (days 1–10) to bilayers of basal and luminal cells (days 10–15) to a vast majority of luminal cells (adult tissue) (10). Thus we colocalized the connexins with specific cell markers for basal and luminal

epithelial cells. At day 6 and 10, Cx43 colocalized with cytokeratins 5/15, which are markers of basal epithelial cells (Fig. 2A). In day 90 prostates, Cx32 colocalized to cells containing cytokeratins 8/18 (Fig. 2B), which are markers of differentiated luminal cells. Of interest, a sparse number of basally located cells were not stained by either antibody. These data confirm that Cx43 is the gap junction protein in prostatic basal cells whereas Cx32 is the gap junction protein of differentiated luminal cells.

Lastly, to characterize the connexin stain observed in the stroma, we performed colocalization studies for either Cx43 or Cx32 with α-actin as a marker for differentiated smooth muscle cells. Because α-actin first appears in the developing ventral prostate around day 3 (10), we examined days 4, 5, 6, and 10 for Cx43 and α-actin and days 15 and 30 for Cx32 and α-actin. At days 4–6, Cx43 primarily localized to periurethral mesenchymal cells that did not stain for α-actin (Fig. 2, C and D). Differentiating periductal smooth muscle cells

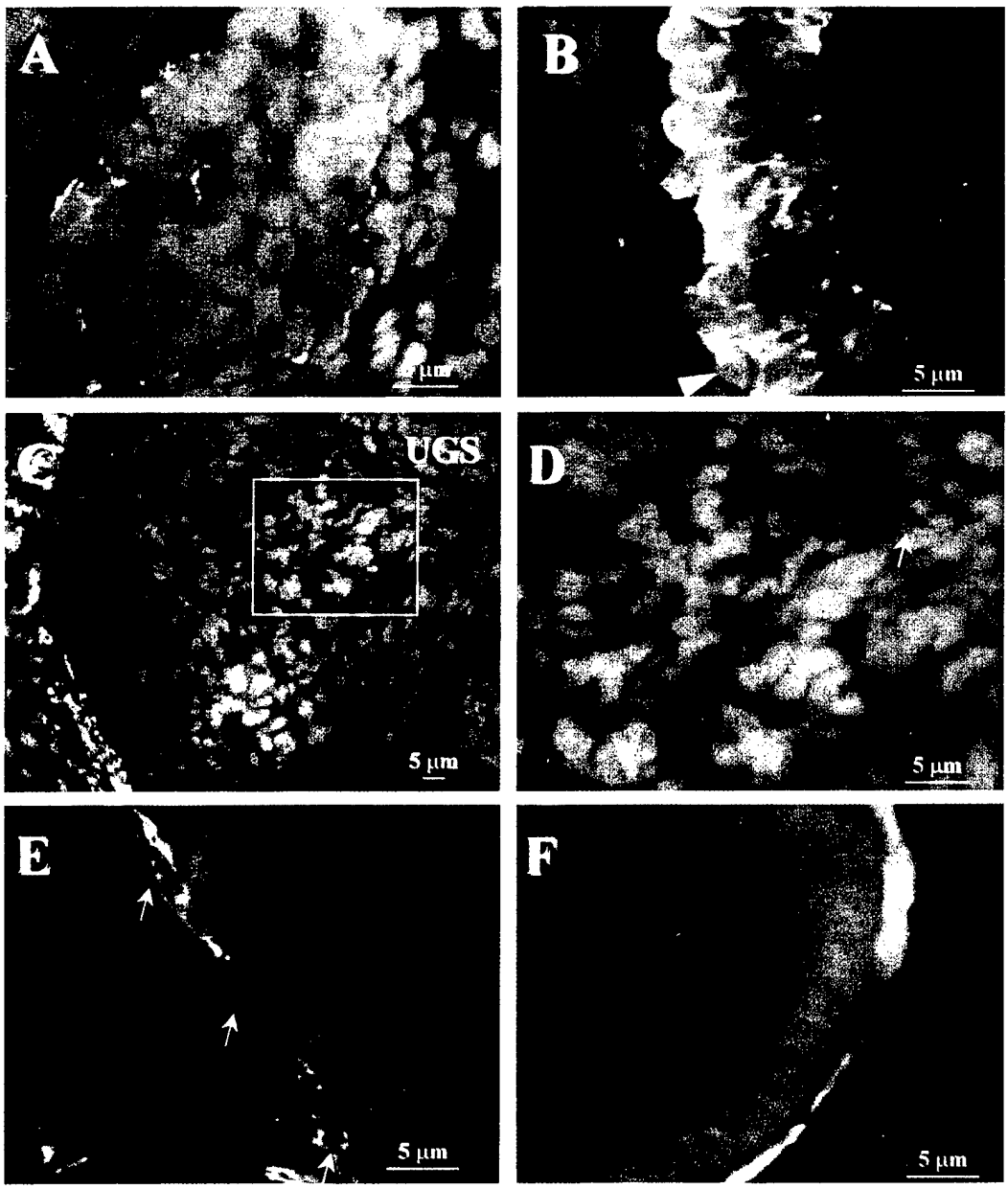

FIG. 2. Double-label immunofluorescent staining of Cx43 and Cx32 with cell markers in the normal rat ventral prostate. A, Day 6 ventral prostate labeled with Cx43 (*red*) and cytokeratins 5/15 (*green*) illustrates that basal cells alone express Cx43. B, Day 90 ventral prostate labeled with Cx32 (*red*) and cytokeratins 8/18 (*green*) illustrates that Cx32 localizes to luminal cells. Arrowhead points to a basal cell that stains for neither antigen. C, Day 5 UGS mesenchyme immunolabeled for Cx43 (*red*) and smooth muscle α-actin (*green*). Note that the red Cx43 label localizes to the periurethral mesenchyme proximal to the smooth muscle sleeve surrounding the UGS. D, Higher power view of boxed image in C shows that mesenchymal but not smooth muscle cells express Cx43 the cell-cell contact areas (*arrows*). E, Day 15 ventral prostate immunolabeled for Cx32 (*red*) and α-actin (*green*) reveal that smooth muscle cells surrounding the acini form gap junctions composed of Cx 32 (*arrows*). F, Day 30 ventral prostate immunolabeled for Cx32 (*red*) and α-actin (*green*) show intense Cx32 along epithelial borders and limited Cx32 in periductal smooth muscle cells.

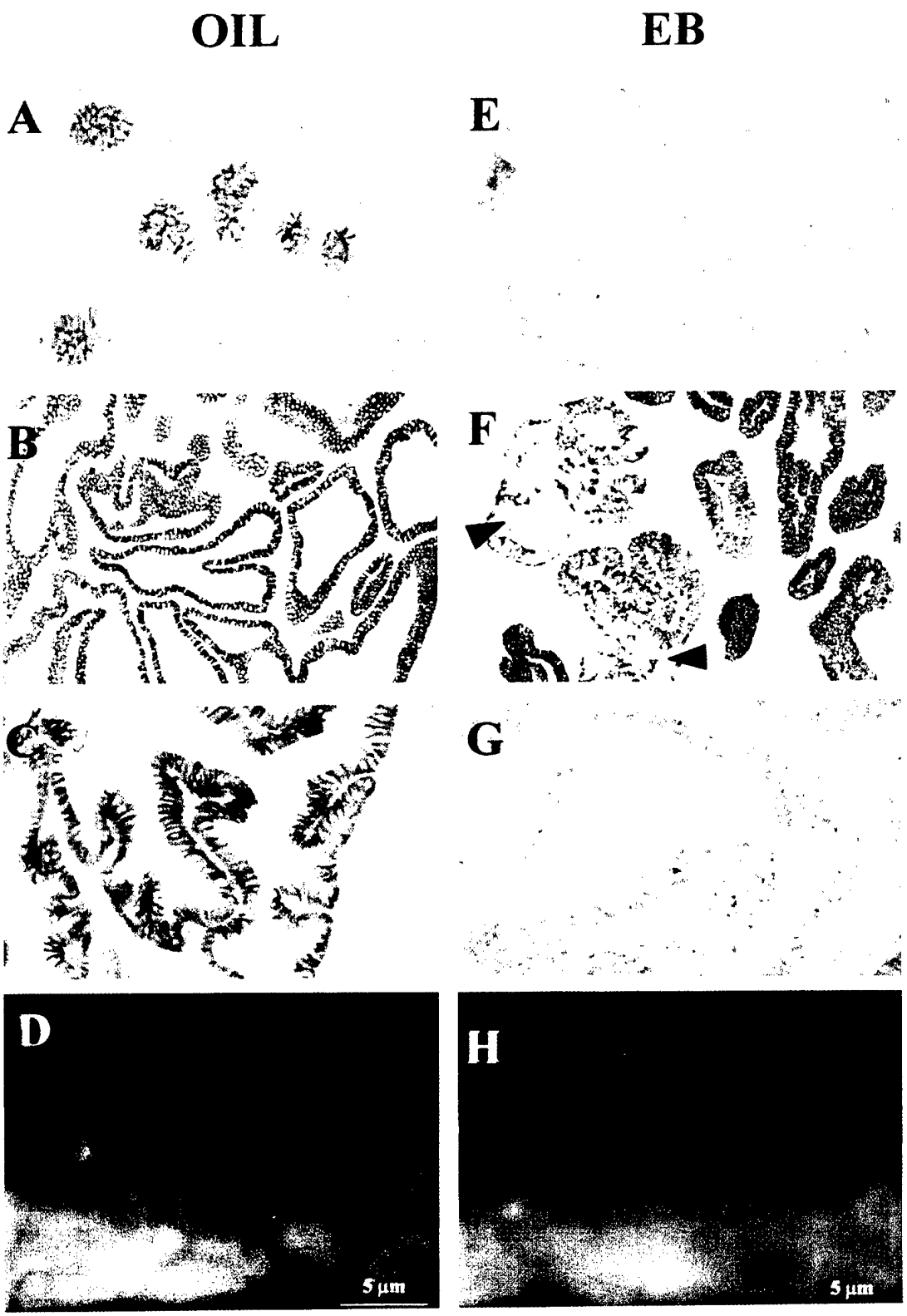

FIG. 3. Immunostaining for E-cadherin in ventral prostates of oil-treated (A–D) and neonatally estrogen-treated (E–H) rats. A, Day 6 ventral prostate of oil-treated rat shows E-cadherin along the lateral surfaces of epithelial cells in the solid epithelial buds (×100). B, At day 30, all luminal epithelial cells of the normal prostate show intense stain for E-cadherin along the lateral borders (×50). C, In the aged ventral prostate (16 months), epithelial cells continue to immunostain for E-cadherin (×100). D, Double label immunofluorescent stain for E-cadherin (*green*) and Cx32 (*red*) in the day 90 ventral prostate illustrates that they colocalize to the lateral borders of the same epithelial cells. *Yellow* stain is observed when the two proteins colocalize to the same spot. E, Day 6 estrogen-treated ventral prostate immunostained for E-cadherin on the same glass slide as control tissue shown in A. E-cadherin localizes to the cell borders of undifferentiated epithelium, although the intensity

in the proximal regions of the prostate colocalized with Cx43; however, this did not extend past the smooth muscle sleeve surrounding the UGS. At day 15, a small number of periductal smooth muscle cells colocalized α-actin and Cx32, however, this disappeared by day 30 (Fig. 2, E and F).

2) Ontogeny of E-cadherin in rat prostate

Undifferentiated epithelial cells of the developing prostate gland stained positive for E-cadherin at cell-cell contact areas (Fig. 3A). The staining intensity markedly increased between days 10–15 when epithelial cells of the ventral, dorsal and lateral lobes began to differentiate into a luminal cell phenotype. Luminal cell staining increased further between days 15–30 as the majority of epithelial cells became functionally differentiated (Fig. 3B). In the adult day 90 rat prostate lobes, intense staining for E-cadherin was found along the lateral borders of all luminal epithelial cells, and this expression continued in the aged (16 months) prostates where intense staining was observed in both cuboidal and columnar epithelial cells (Fig. 3C). Colocalization studies in the day 90 prostate revealed that Cx32 and E-cadherin colocalized to the same luminal epithelial cells but not always to the same spots on the cell membrane (Fig. 3D).

3) Effects of neonatal estrogenization on connexin and E-cadherin expression

Neonatal exposure to estrogens did not have an immediate effect on the epithelial cell connexin expression in the developing prostate lobes examined between days 5–30. As determined by colocalization studies of connexins with cytokeratins, undifferentiated and basal epithelial cells contained gap junctions comprised solely of Cx43, whereas differentiated luminal epithelial cells between days 15–30 contained gap junctions composed of Cx32 proteins. However, by day 30, Cx32 appeared disorganized in epithelial cells of the ventral prostate with localization throughout the cytoplasm (Fig. 4A) in contrast to the distinct membrane localization of control ventral lobes (Fig. 2F). Our previous studies have found that neonatal estrogen has lobe-specific effects on the adult prostate (40). While all lobes show a growth inhibition in response to early estrogen exposure, epithelial hyperplasia and dysplasia are most prominent in the ventral prostate with moderate effects observed in the dorsal lobe. In contrast, the lateral lobes do not exhibit epithelial distortion and dysplasia with aging following a brief exposure to estrogens during the neonatal period. To determine whether the epithelial differentiation defects and development of dysplasia were associated with alterations in Cx 32, Cx 43, and E-cadherin levels, we focused on the ventral prostate lobes of young adult and aging ventral prostates in the present study. Adult ventral prostates of neonatally estrogenized rats exhibited a marked decrease in Cx32 staining (Fig. 4, B and E) compared with oil-treated controls (Fig. 1F). At the same time, there was an increased proportion of Cx43 expressing cells in the adult ventral prostate lobes (Fig. 4C) in contrast to the infrequent Cx43 positive cells in control prostates (Fig. 4D). The observed higher number of Cx43-expressing basal cells were most noticeable in the proximal and central regions of the estrogenized ducts upon examination of longitudinal sections. This shift in connexin protein profiles in prostate epithelia corresponds to the known accumulation of undifferentiated basal-type cells in response to neonatal estrogen exposure (10). However, a change in the proportions of epithelial cell types does not alone explain the alterations in connexin profiles in the estrogenized prostates. Colocalization of Cx32 and cytokeratin 8/18 (luminal cell marker) in estrogenized prostates revealed a decrease of Cx32 expression in luminal cells compared with controls (Fig. 4E), particularly in hyperplastic areas (Fig. 4F), whereas there was a complete loss of Cx32 in dysplastic epithelia.

Neonatal estrogen exposure also affected Cx43 expression in the mesenchymal cells of the developing prostate gland. In addition to heavy Cx43 staining in the day 10 periurethral mesenchymal cells of the UGS (Fig. 4G), estrogenized prostates contained Cx43-expressing cells into the proximal regions of the ventral prostate mesenchymal pad (*i.e.* beyond the smooth muscle sleeve). Colocalization of Cx43 with α-actin revealed that this proximal Cx43 staining primarily localized to actin-negative, periductal fibroblasts (Fig. 4H) and only occasionally to differentiating smooth muscle cells. Colocalization studies of Cx32 and α-actin showed no expression of Cx32 in smooth muscle cells of the day 15 and 30 estrogenized prostates and instead occasional Cx32 staining in fibroblast cells (Fig. 4A).

E-cadherin immunostaining was present but less intense in the undifferentiated epithelial cells of day 6–10 estrogenized prostates when compared with oil-treated controls (Fig. 3E). As epithelial cells in the central to distal regions of the gland differentiated between days 15–30, strong E-cadherin stain was observed similar to control tissues. However, as early as day 30, small foci of epithelial cells which were E-cadherin negative, were noticeable within the ducts of estrogenized ventral prostates (Fig. 3F). In day 90 and 16-month-old ventral prostates, larger foci with differentiation defects and dysplasia were associated with loss or decrease in E-cadherin immunostaining in the epithelium (Fig. 3G). Colocalization of E-cadherin with Cx32 revealed that in normal appearing regions of neonatally estrogenized adult prostates both Cx32 and E-cadherin were reduced (Fig. 3H), whereas dysplastic glands expressed no Cx32 and only very low E-cadherin.

Discussion

Gap junction proteins have not been previously described in the developing prostate gland. In the present study, we found Cx43 but not Cx32 immunostain in undifferentiated

of immunostaining is reduced. (×100). F, Day 30 estrogenized ventral prostate shows E-cadherin along the cell lateral surface of most epithelial cells; however, there are small foci of epithelium that do not stain for E-cadherin (*arrowheads*) (×50). G, Aged ventral prostate from an estrogenized rat contains large areas of acini which contain dysplastic epithelial cells that express little or no E-cadherin. (×100). H, Double-label immunofluorescent stain of E-cadherin (*green*) and Cx32 (*red*) in epithelium of day 90 estrogenized prostate. Both E-cadherin and Cx32 are reduced when compared with oil-treated control prostates.

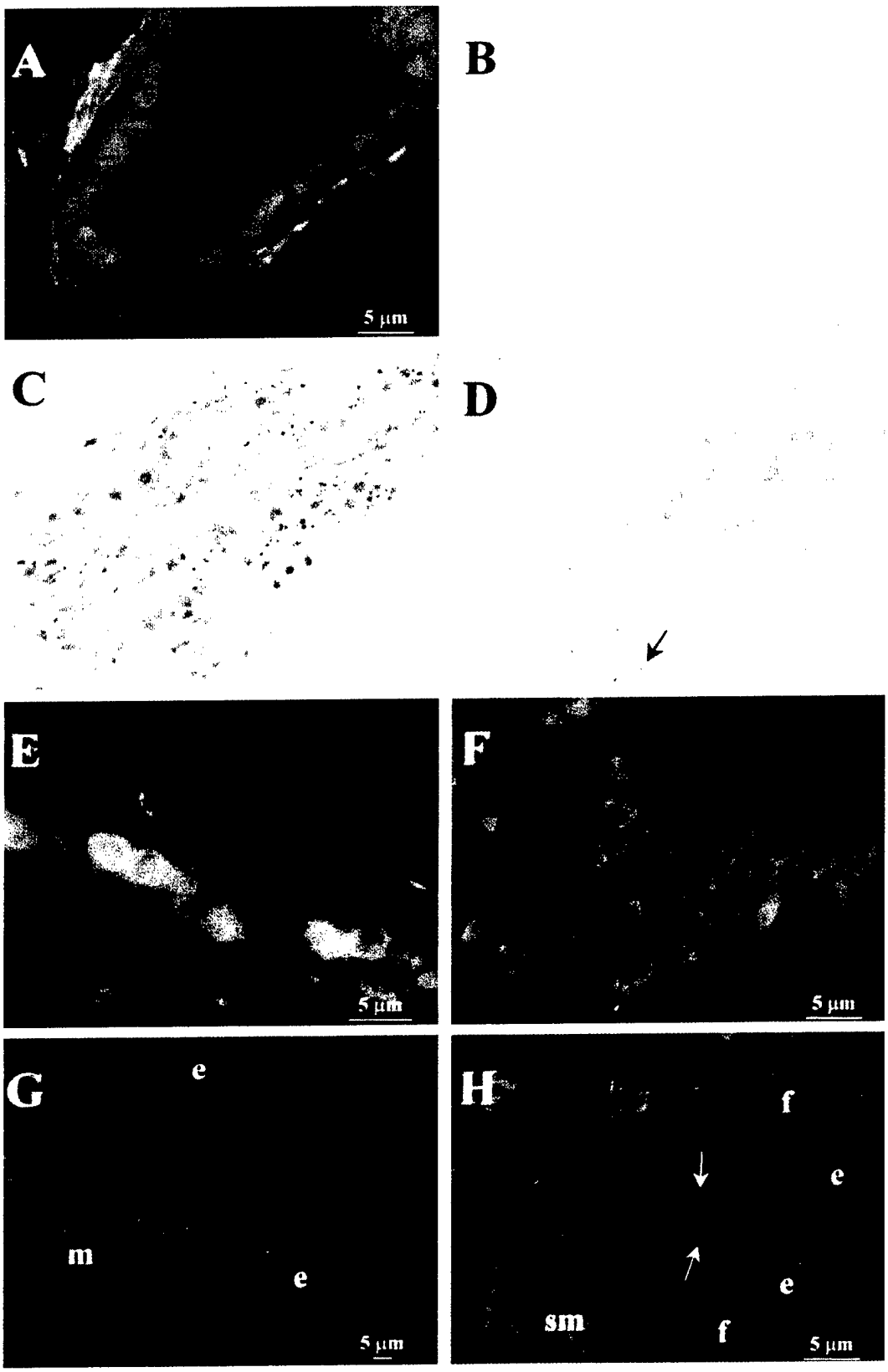

FIG. 4. Immunocytochemistry and immunofluorescence of Cx43 and Cx32 in prostates from neonatally estrogenized rats. A, Double-label immunofluorescent stain of Cx32 (*red*) and α-actin (*green*) illustrates that epithelial and stromal cells of the day 30 estrogenized prostate express Cx32; however, the stain appears in aggregates throughout the cytoplasm. In the stroma, Cx32 stain is not within the smooth muscle (α-actin positive) cells but rather within fibroblast cells. B, Immunostain for Cx32 in day 90 estrogenized ventral prostates reveals a marked decrease in epithelial cell immunolabeling (×200). C, Day 90 estrogenized ventral prostate immunostained for Cx43 shows that a high number of epithelial

epithelial cells beginning at day 1 of life with increasing Cx43 stain intensity during the first 10 days. This molecule was localized throughout the lengthening ducts with an increasing expression gradient from the UGS out to the distal tips. This gradient can be explained by our previous observation that epithelial cell differentiation and ductal lumenization begin in the proximal ducts and spread toward the distal tips during prostate morphogenesis (10). In the present study, as the epithelial ducts began to luminize, the expression of Cx43 declined along with a decline in the number of basal-type undifferentiated cells. Concomitantly, there was an increase in the differentiated luminal cell population which expressed Cx32. We thus conclude that basal and luminal cells communicate via GJ composed of specific GJ proteins; basal cells via GJ composed of Cx43 and luminal cells via GJ composed of Cx32. This was further confirmed by the results of our colocalization studies showing that Cx43 colocalized with cell markers for undifferentiated basal cells and Cx32 with markers for differentiated luminal cells and both GJ-proteins never colocalized to the same cells. Apparently, basal and luminal epithelial cells do not communicate through these two investigated connexins. These findings are in accordance with reports of Cx32 expression but lack of Cx43 expression in adult rat prostates (24), the ducts of which are almost entirely composed of luminal cells. Similarly, the present findings that E-cadherin expression increases with age in the developing rat prostate is in accordance with the differentiation of epithelial cells into a luminal cell phenotype and functional differentiation from day 15–30 onwards. In the human prostate, luminal cells express E-cadherin while basal cells express E- and P (placenta)-cadherin (41).

Mesenchymal cells of the UGS and basal cells of the developing prostate ducts expressed Cx43 during the same period (day 1-day 15). Mesenchymal Cx43-expression was strong at birth in periurethral mesenchymal cells and did not extend past the smooth muscle sleeve surrounding the UGS into the prostate proper in normal control rats. As the process of morphogenesis continued, Cx43 expression in periurethral stromal cells was lost suggesting that it is only expressed in a subset of undifferentiated mesenchymal cells. In turn, Cx32 was observed in differentiated smooth muscle cells for a limited period of time during morphogenesis. In those cells, Cx32 expression was highest at day 15, declined markedly by day 30 and was not found in the adult prostate smooth muscle cells. These findings suggest that mesenchymal and stromal cells communicate through these specific gap junctions during the period of morphogenesis only. While reports on connexin expression in mesenchymal cells are limited, both Cx43 and Cx32 have been identified in mesenchymal cells of fetal chicks and mice (42, 43). Interestingly, in the developing chick limb buds, high Cx43 expression in the ectodermal ridge has been shown to induce gap junction expression in distal mesenchymal cells (34).

For both epithelial and mesenchymal cells, the "switch" from Cx43 to Cx32 expression appears to be associated with differentiation. Developmental "switches" in the expression of GJ-protein subclasses are reported in other cell systems and are associated with maturation processes (44, 45). GJIC is believed to provide a mechanism for coordination of cellular activities in "developmental compartments." The down-regulation of a particular connexin may reflect an early change in metabolic activity within a certain compartment associated with subsequent quiescence of these cells (14). In this sense, the decreased expression of Cx43 may reflect a decreased metabolic activity of the basal and mesenchymal cell population, whereas the increase in Cx32-expression with age could be an expression of increased activity in the luminal cell compartment and a transient increased activity in differentiated smooth muscle cells.

Developmental estrogenization

Following neonatal exposure to estradiol, there were no immediate alterations noted in Cx43 immunostaining in undifferentiated basal cells. Similarly, when epithelial cells began to show evidence of differentiation into luminal epithelial cells, albeit at a delayed rate, Cx32 immunostaining was present in those cells. The first observed difference in epithelial connexin expression noted in estrogenized prostates was at day 30 when Cx32 was localized within the cytoplasm in contrast to the distinct membrane-associated localization observed in control prostates. Similar alterations in Cx32 redistribution to the cytoplasm from the membrane have been reported for human prostate cancers (25). Interestingly, in moderately differentiated carcinomas, Cx32 immunostaining was frequently localized to the cytoplasm, whereas in poorly differentiated cancers, there was loss of Cx32 expression altogether. It has been noted that neoplastic cells have fewer GJ compared with homologous nonneoplastic cells and reduced GJIC compared with their normal counterparts (16). Likewise, the restoration of GJIC in neoplastic cells by means of overexpression of connexin genes has resulted in abrogation of neoplastic growth and tumorigenicity (16, 25, 46). In the present study, aging of the estrogenized rat prostates resulted in a decrease in Cx32 expression in the luminal epithelial cells of the ventral lobe, which was most pronounced in areas of hyperplasic and dysplastic epithelium. The cause of reduced Cx32 expression may relate to the loss of androgen receptor expression in permanently imprinted ventral prostate epithelial cells (3). Although androgen regulation of connexins has not been established for the prostate gland, testosterone has been shown to increase Cx32 expression in spinal cord motorneurons (16).

In addition to altered Cx32 levels, there was a significant increase in the number of epithelial cells expressing Cx43 in adult estrogenized ventral prostates, which correlates with

cells express Cx43 in contrast to the infrequent Cx43 positive cells in oil-treated control prostates, D (×200). E, Double-label for Cx32 (*red*) and cytokeratins 8/18 (*green*) of day 90 estrogenized prostate shows a decrease in Cx32 expressing cells throughout the epithelium and only occasional Cx32 expressing cells in hyperplastic epithelium, F. G, Double-label for Cx43 (*red*) and α-actin (*green*) in the day 10 estrogenized UGS shows intense Cx43 labeling throughout the mesenchymal (m) and differentiating smooth muscle cells. H, Day 10 estrogenized ventral prostate immunolabeled for Cx43 (*red*) and α-actin (*green*) shows that in the proximal region of the prostate gland proper, Cx43 (*arrows*) is expressed by periductal fibroblast (f) cells (sm, smooth muscle; e, proximal epithelial duct).

the previously documented accumulation of basal cells in that tissue (10). In the normal adult prostate, epithelial cells expressing Cx43 are infrequent (24); thus, the presence of large numbers of Cx43-expressing epithelial cells in estrogenized adult prostates could reflect a "switch" toward dedifferentiation. Similar findings were observed in immortalized mouse hepatocytes, where the expression of Cx43 correlated with dedifferentiation of those cells (47). Thus, it is possible that the estrogen-induced differentiation defects, hyperplasia and dysplasia occurring with maturation and aging of the ventral prostate may be mediated in part via alterations in Cx32 and Cx43 resulting in aberrant cell-cell communication.

In contrast to the seemingly delayed effect of estrogen exposure on epithelial connexin expression, there was an early alteration in mesenchymal/stromal connexin expression at the time of estrogen exposure. Thus there was increased expression of Cx43 in periurethral mesenchymal cells as well as an extension of Cx43-positive cells beyond the periurethral smooth muscle sleeve into the proximal regions of the prostatic mesenchymal pad. We have previously reported that neonatal estrogen treatment leads to immediate proliferation of prostatic periductal fibroblasts, particularly within the proximal ductal regions thus creating a physical barrier between smooth muscle and epithelial cells, which constrains branching morphogenesis and blocks the essential paracrine communications which normally control morphogenesis (12). The increased GJIC via gap junctions composed of Cx43 in periductal fibroblastic cells in estrogenized prostates may reflect an increase in metabolic activity needed for their proliferation. We have also shown that neonatal estrogenization results in an immediate up-regulation of ERα in periductal stromal cells along the length of the developing prostatic ducts. Since evidence has been presented that estrogens can directly regulate Cx43 expression (28, 29), the present findings would indicate that Cx43 up-regulation in mesenchymal cells may be directly mediated via the mesenchymal/stromal ERα.

Neonatal estrogen exposure also resulted in an early decrease in prostatic epithelial E-cadherin staining intensity at day 6 when directly compared with control prostates, suggesting apposing effects of neonatal estrogen exposure on Cx43 and E-cadherin expression. By day 30, E-cadherin negative epithelial foci were observed, whereas in the aged estrogenized rats, E-cadherin was reduced across all epithelium in general and was absent in dysplastic epithelial cells. When colocalizing Cx32 and E-cadherin in aged estrogenized ventral prostates, reduced expression of both molecules was noted in normal appearing regions, whereas epithelial dysplasia was associated with a complete loss of Cx32 and very low E-cadherin staining. These data are in accordance with previously reported differentiation defects in estrogenized epithelium, particularly in the ventral lobe. Cell-cell adhesion and communication are intimately related; cells expressing connexins can assemble GJ only when cadherins are also expressed (14, 48, 49). E-cadherin was reported to function as a tumor suppressor protein, particularly as a suppressor of tumor cell invasion (36). E-cadherin expression is decreased in high grade prostate cancer and decreased E-cadherin expression is causally related to prostate cancer progression and invasiveness (33, 50, 51). As decreased expression of E-cadherin is associated with dedifferentiation, invasion and metastatic potential of tumor cells and perturbed GJIC is implicated in carcinogenesis, these dysplastic foci could represent the preinvasive state of transformed epithelial cells.

In conclusion, we found evidence that Cx43 is expressed in undifferentiated and mature basal cells as well as UGS mesenchymal cells, whereas Cx32 is expressed by differentiated prostate epithelial cells and E-cadherin expression increases with age and differentiation. Thus expression of Cx32 and E-cadherin may mark the differentiated state, and that of Cx43 the undifferentiated proliferating state in the prostate epithelium. Neonatal estrogenization of the prostate increases Cx43 and decreases Cx32 as well as E-cadherin expression, which may indicate a "switch" from differentiation to epithelial dedifferentiation. Estrogen-induced changes in the expression of E-cadherin and Cx32 and Cx43, and their assembly into GJs may result in defective cell-cell communication and impaired cell-cell adhesion, and may be one of the key mechanisms through which changes toward a dysplastic state are mediated.

References

1. **Rajfer J, Coffey DS** 1978 Sex steroid imprinting of the immature prostate. Invest Urol 16:186–190
2. **Naslund MJ, Coffey DS** 1986 The differential effects of neonatal androgen, estrogen and progesterone on adult rat prostate growth. J Urol 136:1136–1140
3. **Prins GS** 1992 Neonatal estrogen exposure induces lobe-specific alterations in adult rat prostate androgen receptor expression. Endocrinology 130:3703–3714
4. **Prins GS, Woodham C, Lepinske M, Birch L** 1993 Effects of neonatal estrogen exposure on prostatic secretory genes and their correlation with androgen receptor expression in the separate prostate lobes of the adult rat. Endocrinology 132:2387–2398
5. **Prins GS** 1997 Developmental estrogenization of the prostate gland. In: Naz RK (ed) Prostate: Basic and Clinical Aspects. CRC Press, Boca Raton, FL, Chapter 10, pp 247–265
6. **Pylkkanen L, Makela S, Valve E, Harkonen P, Toikkanen S, Santti R** 1993 Prostatic dysplasia associated with increased expression of C-MYC in neonatally estrogenized mice. J Urol 149:1593–1601
7. **Santti R, Newbold RR, Makela S, Pylkkanen L, McLachlan JA** 1994 Developmental estrogenization and prostatic neoplasia. Prostate 24:67–78
8. **Prins G, Birch L** 1997 Neonatal estrogen exposure up-regulates estrogen receptor expression in the developing and adult rat prostate lobes. Endocrinology 138:1801–1809
9. **Prins G, Marmer M, Woodham C, Chang W, Kuiper G, Gustafsson J, Birch L** 1998 Estrogen receptor-β messenger ribonucleic acid ontogeny in the prostate of normal and neonatally estrogenized rats. Endocrinology 139:874–883
10. **Prins GS, Birch L** 1995 The developmental pattern of androgen receptor expression in rat prostate lobes is altered after neonatal exposure to estrogen. Endocrinology 136:1303–1314
11. **Chang WY, Birch L, Woodham C, Gold LI, Prins GS** 1999 Neonatal estrogen exposure alters the transforming growth factor-β signaling system in the developing rat prostate and blocks the transient $p21^{cip1/wafl}$ expression associated with epithelial differentiation. Endocrinology 140:2801–2813
12. **Chang WY, Wilson MJ, Birch L, Prins GS** 1999 Neonatal estrogen stimulates proliferation of periductal fibroblasts and alters the extracellular matrix composition in the rat prostate. Endocrinology 140:405–415
13. **Garrod D, Chidgey J, North A** 1996 Desmosomes: differentiation, development, dynamics and disease. Curr Opin Cell Biol 8:670–678
14. **Bruzzone R, White T, Paul D** 1996 Connections with connexins: the molecular basis of direct intercellular signaling. Eur J Biochem 238:1–27
15. **Guthrie S, Gilula N** 1989 Gap junctional communication and development. Trends Neurosci 12:12–16
16. **Ruch R** 1994 The role of gap junctional intercellular communication in neoplasia. Ann Clin Lab Sci 24:216–231
17. **Geiger B, Yehuda-Levenberg S, Bershadsky A** 1995 Molecular interactions in the submembrane plaque of cell-cell and cell-matrix adhesions. Acta Anat 154:46–62
18. **Goodenough D, Goliger J, Paul D** 1996 Connexins, connexons, and intercellular communication. Annu Rev Biochem 65:475–502
19. **Aberle H, Schwartz H, Kemler R** 1996 Cadherin-catenin complex: protein interactions and their implications for cadherin function. J Cell Biochem 61:514–523

20. **Barth A, Nathke I, Nelson W** 1997 Cadherins, catenins and APC protein: interplay between cytoskeletal complexes and signaling pathways. Curr Opin Cell Biol 5:683–690
21. **Caveney S** 1985 The role of gap junctions in development. Annu Rev Physiol 47:319–335
22. **Yancey S, Biswal S, Revel J** 1992 Spatial and temporal patterns of distribution of the gap junction protein connexin43 during mouse gastrulation and organogenesis. Development 114:203–212
23. **Ruangvoravat C, Lo C** 1992 Connexin 43 expression in the mouse embryo: localization of transcripts within developmentally significant domains. Dev Dynam 194:261–281
24. **Meda P, Pepper M, Traub O, Willecke K, Gros D, Beyer E, Nicholson B, Paul D, Orci L** 1993 Differential expression of gap junction connexins in endocrine and exocrine glands. Endocrinology 133:2371–2378
25. **Mehta PP, Perez-Stable C, Nadji M, Mian M, Asotra K, Roos BA** 1999 Suppression of human prostate cancer cell growth by forced expression of connexin genes. Dev Genet 110:91–110
26. **Mehta PP, Lokeshwar BL, Schiller PC, Bendix MV, Ostenson RC, Howard GA, Roos BA** 1996 Gap-junctional communication in normal and neoplastic prostate epithelial cells and its regulation by cAMP. Mol Carcinog 15:18–32
27. **Tsai H, Werber J, Davia M, Edelman M, Tanaka K, Melman A, Christ G, Geliebter J** 1996 Reduced connexin 43 expression in high grade, human prostatic adenocarcinoma cells. Biochem Biophy Res Commun 227:64–69
28. **Yu W, Dahl G, Werner R** 1994 The connexin43 gene is responsive to oestrogen. Proc R Soc Lond B 255:125–132
29. **Lye S, Nicholson B, Mascarenhas M, MacKenzie L, Petrocelli T** 1993 Increased expression of connexin-43 in the rat myometrium during labor is associated with an increase in the plasma estrogen:progesterone ratio. Endocrinology 132:2380–2386
30. **Piersanti M, Lye S** 1995 Increase in messenger ribonucleic acid encoding the myometrial gap junction protein, connexin-43, requires protein synthesis and is associated with increased expression of the activator protein-1 c-*fos*. Endocrinology 136:3571–3578
31. **Bennett M, Barrio L, Bargiello T, Spray D, Hertzberg E, Saiz J** 1991 Gap junctions: new tools, new answers, new questions. Neuron 6:305–320
32. **Gilbert SF** 1994 Developmental Biology, ed 4. Sinauer Associates, Sunderland, MA
33. **Bussemakers M, van Moorselaar R, Giroldi L, Ichikawa T, Isaacs J, Takeichi M, Debruyne F, Schalken J** 1992 Decreased expression of E-cadherin in the progression of rat prostatic cancer. Cancer Res 52:2916–2922
34. **Green C, Bowles L, Crawley A, Tickle C** 1994 Expression of the connexin43 gap junctional protein in tissues at the tip of the chick limb bud is related to the epithelial-mesenchymal interactions that mediate morphogenesis. Dev Biol 161:12–21
35. **Kleinerman D, Troncoso P, Lin S, Pisters L, Sherwood E, Brooks T, von Eschenbach A, Hsieh J** 1995 Consistent expression of an epithelial cell adhesion molecule (C-CAM) during human prostate development and loss of expression in prostate cancer: implication as a tumor suppressor. Cancer Res 55:1215–1220
36. **Vleminckx K, Vakaet Jr L, Mareel M, Fiers W, Van Roy F** 1991 Genetic manipulation of e-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell 66:107–119
37. **Rose B, Mehta P, Loewenstein W** 1993 Gap junction protein gene suppresses tumorigenicity. Carcinogenesis 14:1073–1075
38. **Kleinerman D, Zhang W, Lin S, Van N, von Eschenbach A, Hsieh J** 1995 Application of a tumor suppressor (C-CAM1)-expressing recombinant adenovirus in androgen-independent human prostate cancer therapy: a preclincial study. Cancer Res 55:2831–2836
39. **Prins GS, Birch L, Greene GL** 1991 Androgen receptor localization in different cell types of the adult rat prostate. Endocrinology 129:3187–3199
40. **Greene D, Wheeler T, Egawa S, Weaver R, Scardino P** 1991 Relationship between clinical stage and histological zone of origin in early prostate cancer: morphometric analysis. Br J Urol 68:499–509
41. **Shimoyama Y, Hirohashi S, Hirano S, Noguchi M, Takeichi M, Abe O** 1989 Cadherin cell adhesion molecules in human epithelial tissues and carcinomas. Cancer Res 49:2128–2133
42. **Makarenkova H, Becker D, Tickle C, Warner A** 1997 Fibroblast growth factor 4 directs gap junction expression in the mesenchyme of the vertebrate limb bud. J Cell Biol 138:1125–1137
43. **Allen F, Tickle C, Warner A** 1990 The role of gap junctions in patterning of the chick limb bud. Development 108:623–634
44. **Risek B, Klier F, Gilula N** 1992 Multiple gap junction genes are utilized during rat skin and hair development. Development 116:639–651
45. **Risek B, Klier F, Gilula N** 1994 Developmental regulation and structural organization of connexins in epidermal gap junctions. Dev Biol 164:183–196
46. **Mehta PP, Hotz-Wagenblatt A, Rose B, Shalloway D,Loewenstein WR** 1991 Incorporation of the gene for a cell-cell channel protein into transformed cells leads to normalization of growth. J Membr Biol 124:207–225
47. **Stutenkemper R, Geisse S, Schwarz H, Look J, Traub O, Nicholson B, Willecke K** 1992 The hepatocyte-specific phenotype of murine liver cells correlates with high expression of connexin32 and connexin26 but very low expression of connexin43. Exp Cell Res 201:43–54
48. **Fagotto F, Gumbiner B** 1996 Cell contact-dependent signaling. Dev Biol 180:445–454
49. **Musil L, Cunningham B, Edelman G, Goodenough D** 1990 Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. J Cell Biol 111:2077–2088
50. **Giroldi L, Schalken J** 1993 Decreased expression of the intercellular adhesion molecule E-cadherin in prostate cancer: biological significance and clinical implications. Cancer Metastasis Rev 12:29–37
51. **Umbas R, Schalken J, Aalers T, Carter B, Karthaus E, Schaafsma H, Debruyne F, Isaacs W** 1992 Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. Cancer Res 52:5104–5109
52. **Goodenough D, Paul D, Jesaitis L** 1988 Topological distribution of two connexin 32 antigenic sites in intact and split rodent hepatocyte gap junctions. J Cell Biol 107:1817–1824
53. **Mehta P, Yamamoto M, Rose B** 1992 transcription of the gene for the gap junctional protein connexin 43 and expression of functional cell-to-cell channels are regulated by cAMP. Mol Biol Cell 3:839–850

Impaired Trafficking of Connexins in Androgen-independent Human Prostate Cancer Cell Lines and its Mitigation by α-Catenin

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Running Title: Impaired connexin trafficking in prostate cancer progression

Key words: Gap Junctions, Connexins, Connexin43, Connexin32, Trafficking, and Prostate cancer

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Summary

Gap junctions, formed of proteins called connexins, provide a direct intercellular communication pathway for the diffusion of small growth regulatory molecules between cells. Evidence to date points to the role of connexins as tumor suppressor genes. We had previously shown that the expression of connexin43 and connexin32 in an indolent prostate cancer cell line, LNCaP, resulted in the formation of gap junctions and profound growth inhibition (Dev Genet, 24: 91-110, 1999). To elucidate the role of connexins in the progression of prostate cancer from indolent hormone (androgen)-dependent to independent state, we introduced connexin43 and connexin32 into an invasive, androgen-independent prostate cancer cell line, PC-3. Expression of connexin32 and connexin43 in PC-3 cells resulted in their intracellular accumulation. Western blot analysis revealed lack of Triton-insoluble, plaque assembled form and the abundance of Triton-soluble form of both connexins. Immunofluorescence microscopy showed that both connexins accumulated predominantly in the medial- and trans-Golgi and not in the endoplasmic reticulum. In contrast to indolent LNCaP cells, connexins could not be significantly cell surface biotinylated in invasive PC-3 cells, suggesting their impaired trafficking to the cell surface. Transient transfection of α-catenin, a cadherin associated protein, facilitated the trafficking of both connnexin32 and connexin43 to the cell surface and induced gap junction assembly. Intracellular accumulation of connexins was observed in several androgen-independent prostate cancer cell lines upon transient expression of connexinsr. Our results suggest that the impaired trafficking, and not the inability to form gap junctions, is the major cause of communication deficiency in human prostate cancer cell lines.

Introduction

Cell-cell and cell-matrix adhesion is involved not only in maintaining the structural integrity of cells in tissues but also in governing a wide array of cell behavior (1-3). Cell-cell and cell-matrix adhesion molecules frequently cluster at specific contact areas to form cell junctions, such as adherens junctions, tight junctions, desmosomes, and focal adhesion plaques (1-5). Loss of these junctions has been shown to have prominent consequences on cellular growth, differentiation, and apoptosis during neoplastic development in several tumor model systems (1). Recent studies have shown that the expression of cell-cell and cell-matrix adhesion molecules, such as cadherins and their associate proteins, and integrins is decreased in prostate cancer (PCA) cell lines and that impairment or loss in the expression of these molecules is associated with the malignant potential of prostate epithelial cells (1, 6-12). Direct support for the role of cell adhesion molecules in controlling the invasive behavior of PCA cells has come from studies which show that forced expression of α-catenin, an E-cadherin-associated protein, and C-CAM (9,10), into human PCA cell lines mitigates their malignant phenotype. These studies suggest that direct cell-contact-dependent interactions among epithelial cells in prostate tumors are likely to play an important role in PCA progression.

Besides cell-cell and cell-matrix adhesion junctions, epithelial cells also form a highly specialized class of cell junctions called gap junctions, which are membrane appositions that are traversed by clusters of channels through which molecules up to 1 kDa can directly pass between adjoining cells (13). The cell-cell channels are bicellular structures formed by the members of a

family of about 20 related but distinct proteins named connexin(Cx)s, which first assemble into hexamers to form connexons that align and join with connexons in adjacent cells to form cell –cell channels (14-16). In addition to well-documented role of cell-cell communication mediated by gap junctions, commonly referred to as gap junctional communication (GJC), in the maintenance of tissue homeostasis and synchronization of cellular behavior, it has been proposed that altered GJC and/or impaired expression of Cxs may be one of the genetic or epigenetic changes involved in the initiation and progression of neoplasia (13, 17-19). This notion has been well-supported by several independent studies, which show that forced expression of Cxs genes in several Cx-deficient tumor cell lines attenuates their malignant phenotype (20-24) and by the recent study which shows that the knockout transgenic mice for Cx32 gene, a Cx abundantly expressed in liver, develop a higher incidence of age-related liver tumors and become more susceptible to the tumor promoting effect of liver-specific chemical carcinogens (25).

Although a number of tumor suppressor genes and oncogenes have been implicated in the development of PCA, no consistent genetic or epigenetic changes are known to be associated with its initiation and progression. What is clear, however, is that the incidence of PCA increases with age and is characterized by the progression from an indolent, slow-growing, and hormone (androgen)-dependent state to an invasive, hormone-independent state (10,11,26). Thus, identification of cellular and molecular events that play formative roles in driving the expansion and clonal selection of incipient PCA cells from androgen-dependent to androgen-independent state is essential for

understanding PCA progression and designing strategies for its intervention (11,26). Our previous studies showed that compared to normal prostate epithelial cells, GJC in PCA cell lines was either absent or reduced (20) and that forced expression of Cx32 and Cx43 — the two Cxs expressed by the well-differentiated epithelial cells of the prostate — into an indolent, androgen-dependent and Cx-deficient human PCA cell line, LNCaP, inhibited growth, retarded tumorigenicity, and induced differentiation (20). These studies also showed that Cxs were localized at the cell-cell contact areas in epithelial cells of well-differentiated prostate tumors, whereas they began to accumulate intracellularly as the tumors progressed to more invasive and undifferentiated stages with an eventual loss in advanced stages (20). Prostate epithelial cells from most invasive forms of human androgen-independent prostate carcinomas show frequent impairment and/or deletion of cadherins and their associated proteins such as such as α, β, and γ catenins (10, 11, 26). Because bi-directional signaling between cell adhesion molecules and Cxs may be important in initiating the formation of gap junctions, we investigated if expression of Cx43 and Cx32 into an invasive, androgen-independent PCA cell line, PC-3 with deficient cadherin-mediated adhesion due to the deletion of α-catenin gene (7) will abrogate its malignant phenotype in a manner similar to that of LNCaP cells with functional cadherin-mediated adhesion. Our findings show that in contrast to androgen-dependent PCA cell lines, expression of Cx43 and Cx32 in PC-3 and several other androgen-independent cell lines results in the intracellular accumulation of Cxs as a consequence of defective trafficking and that transient expression of α-catenin, a cadherin-associated protein, triggers trafficking and assembly of Cxs into gap junctions .

MATERIALS AND METHODS

Materials

Cell culture media were obtained from GIBCO/BRL (Gaithersburg, MD). Defined fetal bovine serum was from Hyclone Lab (Logan, UT). Tissue culture plastic ware was from Nalge Nunc International(Rochester, NY). Seakem GTG agarose was from FMC Bio Products (Rockland, ME). TRIzol reagent, geneticin (G418), RNA molecular weight markers and FuGene 6 transfection reagent were from GIBCO/BRL (Gaithersburg, MD, USA). Yeast tRNA, poly(A), poly(C) and herring sperm salmon DNA were from Roche Molecular Biochemicals (Indianapolis, IN). Fluorochrome-conjugated secondary antibodies were from JacksonImmuno Research laboratories (West Grove, PA). Ultrapure formamide was from BD Biosciences Clontech Laboratories (Palo Alto, CA). Lucifer Yellow (LY, lithium salt), rhodamine-conjugated dextrans (MW 10,000, fixable), and Alexa 488 and Alexa 594 conjugated mouse and rabbit secondary antibodies were from Molecular Probes (Eugene, OR). The super-signal chemiluminescent substrate was from Pierce Chemical Co. (Rockford, IL). Enhanced chemiluminescent kit (ECL plus) was from Amersham Life Sciences (Arlington Heights, IL). GeneScreen Plus nylon membranes and [^{32}P]dCTP were from New England Nuclear DuPont (Wilmington, DE). Restriction enzymes and pre-stained protein molecular weight markers were from New England Biolabs (Beverly, MA). BCA reagent for protein determination was from Pierce (Rockford, IL).

Stock solutions of the following reagents prepared as described below were stored frozen and diluted to desired concentration immediately prior to use. Brefeldin (BIOMOL, Plymouth Meeting, PA) was prepared in DMSO at 20 mM and used at 20 μM. Monensin (BIOMOL) was prepared in water at 2.5 mM and used at 2.5 μM. Nocodazole (Sigma, St. Louis, MO) was prepared in ethanol at 15mM stock and used at 15 μM.

Cell Culture

Human PCA cell lines PC-3 (ATCC CRL 1740) and LNCaP (ATCC CRL 1740) were grown in RPMI containing 7.5% defined fetal bovine serum in an atmosphere of 5% CO_2/95% air. Stock cultures were maintained in 12 ml RPMI in 75-cm^2 flasks and sub-cultured weekly at 1.5 x 10^5 cells/flask with a medium change at 3 or 4-d interval as previously described (27). LNCaP clones expressing Cx43 and Cx32 stably were isolated and grown in culture medium supplemented with 200 μg/ml G418 (active) as described (20). The growth characteristics and the hormonal dependence of these cell lines/clones have been described previously (20, 27). The retroviral packaging cell lines PA317 (ATCC CRL 9078,) and PG13 (ATCC CRL 10686,) were grown in RPMI containing 10% defined fetal bovine serum as described previously (20,28).

Antibodies and Immunostaining

A rabbit polyclonal antibody, raised against a synthetic peptide representing amino acids 252-270 of rat Cx43, was used as described previously (29). In addition, we also used the following monoclonal antibodies against Cx43: 13-8300 (Zymed laboratories, Inc., San Francisco, CA), which specifically recognizes the unphosphorylated form of Cx43 (30) and C13720 (Transduction Laboratories, Lexington, KY). Hybridoma M12.13 (31), secreting antibody against rat Cx32, was a generous gift of Dr. Dan Goodenough (Harvard University) and a rabbit polyclonal antibody against Cx32 (71-0600) was purchased from Zymed. We also used the following antibodies: monoclonal antibodies against E-cadherin (13-1700, Zymed), N-cadherin (32), α-catenin and ZO-1 (Transduction laboratories), and polyclonal antibodies against ZO1 (Zymed), α-catenin (Sigma), and calreticulin (PA3-900, Affinity Bioreagents, Inc. Golden, CO).

Cells were immunostained after fixing with methanol/acetone, paraformaldehyde and histochoice (depending on the antibody) as described previously (20,27,29). Briefly, 5 x 10^4 cells were seeded in six well clusters containing glass cover slips and allowed to grow to confluence. They were washed 3 times with PBS, fixed 10 min, and immunostained at room temperature with various antibodies. Secondary antibodies (rabbit or mouse) conjugated with fluorescein, CY2, CY3, Texas red, Alexa 488 and Alexa 594 were used as appropriate. Images of immunostained cells were acquired with Leica DMRIE microscope (Leica Microsystems, Wetzler, Germany) equipped with

Hamamatsu ORCA-ER CCD camera (Hamamatsu-City, Japan). For colocalization studies, serial z-sections (0.5 μm) were collected and analyzed using image processing software (Openlab 3.01; Improvision, Inc; Lexington, MA).

Retroviral Vectors and Plasmids

Plasmids containing cDNAs for various Cxs were obtained from several sources as previously described (20,27-29). Retroviral vector LXSN (33) was a generous gift of Dr. Dusty A. Miller (Fred Hutchinson Cancer Center, Seattle, WA). Retroviral vectors containing rat Cx43 and Cx32 in sense orientation were constructed as described (20) and designated LXSNCx43S and LXSNCx32S, respectively. The retroviral 5' long terminal repeat and SV40 virus promoter drive the expression of Cx cDNAs and neomycin phosphotransferase, respectively (33). Plasmids pECFP-ER and pECFP-Golgi, which are targeted to the endoplasmic reticulum (ER) and Golgi, respectively, and plasmids pECFP-N1, pEGFP-N1, pEGFP-N3 and PEYFP-N1 were purchased from BD Biosciences Clontech (Palo Alto, CA). Chimeras of these fluorescent proteins fused to the carboxy termini of Cx43 and Cx32 were constructed according to the standard molecular biology methods. The details of these constructs and their function and assembly into functional gap junctions will be described elsewhere (Guo et al. in preparation). Plasmid pcDNA3-α-catenin (chicken) was constructed by cloning a chicken 3.5 kb Hind III to Xba1 fragment, encompassing the coding range of chicken α-catenin cDNA from pUC19/21 vector, into the Hind III and Xba I site of pcDNA3.

Retrovirus Production and Infection of Cells

Control and recombinant retroviruses harboring Cx cDNAs were produced in an amphotropic packaging cell line PA317 and, to improve the efficiency of retroviral-mediated gene transfer into human cells, in gibbon ape leukemia virus envelope-based packaging cell line PG13 as previously described (20). The titer of recombinant retroviruses produced from the most stable and the best producing PA317 and PG13 clones were 2 x 10^6 and 4 x 10^5 G418-resistant colony forming units (cfu) per ml, respectively, as measured in rat Morris Hepatoma cells (20, 28). PC-3 cells were infected with equivalent titer (4 x 10^5 cfu/ml) of recombinant retroviruses LXSN (Neo control), LXSNCx32S and LXSNCx43S and selected in G418 (400 μg/ml, active) for 2-3 weeks as described previously (20,28). Glass cylinders were used to isolate individual G418-resistant clones, which were expanded, frozen, and maintained in G418 (200 μg/ml).

Treatments

Cells were seeded in 6-cm (5 X 10^4) or 10-cm (2.5 X 10^5) dishes in 4 ml and 12 ml of complete medium, respectively, and treated when near confluent by replenishing with fresh medium containing various reagents at the desired concentration added from stock solutions prepared as described above so that the final concentration of the solvent did not exceed 0.3%. The controls received fresh medium with 0.1-0.3 % of solvent, which did not affect any of the parameters we measured.

Isolation of RNA and Northern Blot Analysis

Total RNA was extracted from two 10-cm dishes of confluent cells using Trizol reagent as described previously (29). Ten to 20 μg of RNA was analyzed on 1% agarose/formaldehyde gels, transferred to nylon filters, pre-hybridized and hybridized with ^{32}P-labeled DNA probes for various Cxs or GAPDH, washed in 0.1 X SSC at 65 ^{0}C for 1-2 h, and the membranes exposed to Fuji-RX X-ray film for 1–24 h. The labeled DNA probes were prepared using gel-purified fragments (100 ng) and a random priming kit (Roche). The probes were labeled to a specific activity of 10^{8}–10^{9} cpm/μg DNA, and used at 10^{6}cpm/ml hybridization buffer.

Western Blot Analysis

Triton X (TX)100 solubility/insolubility of Cxs was assayed essentially as described by Musil and Goodenough (34,35). Preparation of cell lysates and western blot analysis of Cxs was as described previously (20,27) except for the following modifications. After centrifugation at 50,000 x g for 50 min on a tabletop Beckman ultracentrifuge (Model TL-100) to separate TX100-insoluble and -soluble fractions, the TX100-insoluble pellet was dissolved in 500 μl of solublization buffer (70 mM Tris/HCl, pH 6.8, 8 M urea, 2.5 % SDS and 0.1 M DTT). Total, TX100 soluble and insoluble fractions were mixed with 4 x Laemmli buffer to a final concentration of 1 x and boiled at 100 ^{0}C for 5 min (Cx43) or incubated at room temperature for 60 min (Cx32) before separating by SDS-Page.

Detergent (TX-100) Extraction of Connexin43 and Connexin32 in Situ

Cells were seeded in six well clusters containing glass cover slips as described above (see antibodies and immunostaining), allowed to grow to confluence, and were washed once with PBS at room temperature. Half of the cover slips were extracted with 2 ml 1 % TX100 (Weight/weight) in solution B (30 mM HEPES; 140 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 3 mM glucose) containing a cocktail of protease inhibitors for 30 min at 4 ^{0}C with occasional gentle shaking. Control cells were treated identically except for the omission of 1 % TX100. Cells were immunostained with the antibodies against Cx43 and Cx32 as described above (see antibodies and immunostaining).

Cell Surface Biotinylation of Connexins

PC-3 and LNCaP cells (5 x 10^5) were seeded in 10 cm dishes and grown to 80-90 % confluence. The dishes were transferred to a glass plate placed on ice and gently rinsed 3 times with ice cold PBS (pH 8.0). The biotinylation was initiated by incubating the cells in freshly prepared EZ-Link ^{TM} Sulfo-NHS-SS-Biotin reagent (Pierce Chemical Co.) in PBS (0.5mg/ml) for 30 minutes at 4 ^{0}C. The reaction was quenched by rinsing culture dishes 3 times with ice-cold culture medium containing 15mM glycine. Lysis of monolayers, immunoprecipitation of Cxs and the recovery of biotinylated Cxs were done essentially as described by VanSlyke et al. (36,37). The total Cx

(biotinylated and non-biotinylated) and the biotinylated fraction of samples were either incubated in SDS-PAGE sample buffer for 1 hour for Cx32 or boiled 5 minutes for Cx43 and resolved in 1 x SDS-PAGE.

Transient transfection in PC-3 cells

FuGENE 6 Transfection Reagent was used to transfect cells with various plasmids mentioned above according to the manufacturer's instructions. Briefly, cells (5×10^4) were seeded on to glass cover slips in 6 well culture plates (for immunostaining) or 100 mm dishes (for western blotting) containing 2 ml and 12 ml of complete medium, respectively, and incubated at 37 ^{0}C. After 16 h, cells were transfected with various plasmids using 3 μl FuGENE 6 reagent: 1 μg DNA complex in 100 μl of serum free medium. The transfection complex was pre-incubated for 15 minutes at room temperature before transfection. The medium was replaced by fresh medium 5 h after transfection and cells fixed for immunostaining or lysed for western blotting (see above) after 24-48 h.

Communication Assays

Gap junctional communication was assayed either by microinjecting fluorescent tracer Lucifer Yellow (LY; MW 443 Da) as previously described (28, 38) or by scrape-loading method (39). Briefly, LY (lithium salt, Molecular Probes; 5% aqueous solution) was microinjected into test

cells by Eppendorf InjectMan and FemtoJet microinjection systems(models 5271 and 5242, Brinkmann Instrument, Inc. Westbury, NY) mounted on Leica DMIRE2 microscope. The microinjected cells were viewed with the aid of Sony 3 CCD color video camera (Sony Corp., Japan) at a fixed gain, displayed on a color video monitor (Sony Trinitron), and recorded on a time-lapse videocassette recorder (Toshiba, model AG-6720A). The number of fluorescent cells (excluding the injected one) scored approximately 10 min after injection served as an index of junctional transfer.

For scrape loading, cell culture medium from freshly confluent 6-cm dishes was removed and replaced with 2.5 ml medium containing rhodamine-conjugated fluorescent dextrans (MW 10 kDa, 1 mg/ml; fixable) and LY (0.05 %). Cells were then scrape-loaded by making 4 scratches per dish with a sharp razor blade at room temperature, and, after removing medium, washed quickly 3 times with warm PBS, incubated 5 min at room temperature, and fixed with 3.7 % buffered formaldehyde for 15 min. The fixed dishes were again washed 3 times with PBS and fresh PBS containing 50 mM ammonium chloride was added to quench the auto-fluorescence. The cell-cell transfer of LY was observed under fluorescent microscope.

RESULTS

Overexpression of Connexin43 and Connexin32 in PC-3 Cells

Our previous study showed that PC-3 cells communicated poorly, formed few gap junctions, and expressed a low level of Cx43 mRNA and protein and none of the other Cxs (27). We chose PC-3 cells for expressing Cx43 and Cx32 in PC-3 cells because they have been well characterized and shown to be highly invasive and androgen-independent (10,11,26). Connexin43 and Cx32 were expressed in PC-3 cells by infecting them with a control (LXSN) and Cx-harboring (LXSN43S and LXSN32S) recombinant retroviruses and the expression of Cxs was ascertained in several randomly isolated G-418-resistant clones by northern and western blot analysis. Northern blot analysis of total RNA isolated from Cx43- and Cx32-expressing clones showed abundant expression of retrovirally-transcribed 4.4 Kb Cx43 (Figure 1 A, labeled R-Cx43; lanes PC-43-1 to PC-43-4) and Cx32 (blot not shown) mRNAs that were not expressed by the parental (lanes PC-WT) and control clones (lanes PC-NEO-1 to PC-NEO-4). Moreover, parental PC-3 cells as well as all retrovirally-transduced clones, in addition, expressed a low level of endogenous 3 kb human Cx43 mRNA (Figure 1 A, labeled E-Cx43), which was detected only upon overexposure of blots (data not shown). Western blot analysis (Figure 1 B, C) showed that the control cells, namely, the parental PC-3 cells (lane labeled PC-WT) and PC-3 clones isolated after infection with LXSN (lane labeled PC-NEO) expressed neither Cx32 nor Cx43, whereas clones isolated after infecting with LXSN43S and LXSN32S, expressed abundant Cx43 (Figure 1 A, lanes labeled PC-43-1 to 9) and Cx32 (Figure 1 B,

lanes labeled PC-32-1to10). Taken together, the data in figure 1 show that retroviral transduction of Cx32 and Cx43 in PC-3 cells results in the abundant expression of both Cxs at the mRNA and protein level.

Intracellular Accumulation of Overexpressed Connexin43 and Connexin32 in PC-3 cells

To determine if Cx43- and Cx32-expressing PC-3 clones formed gap junctions, we immunostained cells from several clones with the Cx-specific antibodies. Figure 2 shows the typical immunostaining pattern for Cx43 and Cx32 in one such clone. The results showed that a major portion of both Cxs remained localized in the intracellular compartments (Figure 2, rows 1 and 3) and punctate dots characteristic of gap junctional plaques were rarely observed at cell-cell contact areas (Figure 2, arrow in the middle and right panel of top row). Moreover, in many cells intense intracellular immunostaining was observed not only in the paranuclear areas but also through out the cytoplasm (Figure 2, yellow arrows, middle panels, rows 1 and 3) and near the cell surface membrane (not shown). In contrast to PC-3 cells, both Cxs were assembled into gap junctions in indolent, Cx-expressing LNCaP clones and no intracellular accumulation was observed (Figure 2, rows 2 and 4, junctions indicated by arrows). Because similar results were obtained with all other independently isolated PC-3 clones, we chose only 1 clone for each Cx subtype for our further study.

Detergent-Insolubility of Intracellular Connexin32 and Connexin43 in PC-3 cells

A well-characterized biochemical attribute of Cxs upon assembly into gap junctional plaques is their insolubility in TX100 (34-36). To corroborate the immunocytochemical data and to rule the possibility that intracellular accumulation was due to the formation of gap junctions in the intracellular stores, we extracted Cx32- and Cx43-expressing PC-3 cells in situ with 1 % TX100 for 30 min (see methods) before immunostaining with Cx-specific antibodies and also studied the formation of gap junctions by western blot analysis of the TX100-insolubile extracts. The results showed that only a small fraction of total Cx43 (Figure 3A) and Cx32 (Figure 3B) was converted into TX100-insoluble form in invasive PC-3 cells, whereas a major fraction of both Cxs remained TX100-insoluble in LNCaP and RL-CL9 cells (compare lanes labeled T, S, and I under PC-3, LNCaP and RL-CL9), which form abundant gap junctions (29). Moreover, the results also showed (Figure 3C) that in PC-3 cells, nearly all intracellular Cx32- and Cx43-specific immunostaining was lost upon in situ extraction (compare control and extracted in rows 1 and 3) whereas in LNCaP cells there was no effect (compare control and extracted in rows 2 and 4).

Intracellular accumulation of Cx43 and Cx32 in invasive PC-3 cells was not an artifact of overexpression, because androgen-dependent Cx-expressing LNCaP clones seemed to express more Cxs compared to PC-3 clones (see Figure 3) even though equal amount of total protein was analyzed by western blot analysis (see also discussion). The results shown in Figure 3 suggest that in contrast

to LNCaP cells, both Cx32 and Cx43 accumulate intracellularly in invasive PC-3 cells and that intracellularly accumulated Cxs were not assembled into gap junctions ectopically. Moreover, the data also agree well with our previous in vivo studies, which showed intracellular accumulation of Cx43 and Cx32 and/or loss of formation of gap junctions in epithelial cells of aggressive prostate carcinomas (20).

Communication in Connexin-overexpressing PC-3 Clones

To examine whether formation of few immunocytochemically-detectable gap junctions was sufficient to enhance GJC in Cx-expressing PC-3 clones, we studied the junctional transfer of 443-Da fluorescent tracer, Lucifer Yellow (LY), by micro-injection and scrape-loading (see methods). Figure 4 shows the representative photographs of junctional transfer of LY in control and Cx-expressing PC-3 cells and Table 1 summarizes the data from several microinjections in 2 independent experiments. Typically, junctional transfer of LY is seen at the most to1-2 first order neighbors compared to the control cells in which the transfer is either not detected or rarely observed (Figure 4). The data obtained with the microinjection were independently corroborated by scrape-loading method (39), which measures the communication capacity of several hundred cells simultaneously (Figure 4 legend). Similar data were obtained with 3 other control and Cx43- and Cx32-expressing PC-3 clones (data not shown). Taken together, the data suggest that in contrast to indolent Cx-expressing LNCaP clones (20), reintroduction of Cx43 and Cx32 in invasive PC-3 cells

does not enhance communication significantly, consistent with their intracellular communication and formation of only few immunocytochemically-detectable gap junctions.

Sites of Intracellular Accumulation of Connexin43 and Connexin32

We used double immunofluorescence microscopy to examine the subcellular localization of Cx43 and Cx32 in Cx-expressing PC-3 cells. Calreticulin was used as a maker for the ER (40) whereas transient transfection with the plasmid encoding 1, 4 β-galactosyl transferase tagged with cyan fluorescent protein was used as a marker for the medial- and trans-Golgi (41). Figure 5 shows that as judged by the extent of colocalization of Cxs with these organelle-specific markers, a substantial proportion of Cx43 and Cx32 seems to reside in the medial- and trans-Golgi and not in ER.

To corroborate the colocalization data (see Figure 5), we investigated the effect of different pharmacological reagents known to interfere with the protein trafficking on the localization of Cxs. These reagents included: Brefeldin A, which abolishes the anterograde transport from ER to Golgi (42); monensin, which disrupts trans-Golgi network, lysosomes and acidic endosomes (43) and nocodazole, which disperses Golgi cisternal stacks (44). Figure 6shows that brefeldinA treatment nearly abolished the typical Golgi-like paranuclear distribution pattern of Cx32 and Cx43, whereas monensin treatment had no significant effect. Connexin distribution in nocodazole-treated cells was

mainly localized to the membrane fragments scattered through out the cytoplasm. Because these treatments did not significantly change the level of Cx43 and Cx32 (data not shown), these data, combined with those shown in Figure 5, strongly suggest that a major fraction of both Cxs resides in medial- and trans-Golgi compartment and not in ER or post-Golgi compartments.

Cell Surface Biotinylation of Connexins

Besides intense paranuclear staining, we also observed punctate immunostaining that was scattered through out the cytoplasm and near the cell surface (see Figure 2, cells with yellow arrows). These observations prompted us to investigate whether intracellular accumulation of Cxs was due to their inability to traffic from Golgi stacks to the cell surface or internalization and recycling back into the cytoplasmic stores after arrival at the cell surface. To test this notion, Cx-expressing PC-3 and LNCaP cells were subjected to cell surface biotinylation and the biotinylated fraction of Cxs detected by western blot analysis (see Methods). The results (Figure 7) show that Cx32 and Cx43 could be biotinylated and detected readily in LNCaP cells, whereas in PC-3 cells they could not be biotinylated significantly. The failure to detect significant amount of the biotinylated form of Cx32 and Cx43 in PC-3 cells was not due to inefficient cell surface biotinylation of proteins as judged visually by immunofluorescence microscopy using Alexa flour-conjugated Streptavidin (data not shown). These data, combined with those in Figures 5 and 6, strongly suggest that trafficking of Cxs from medial- and trans-Golgi to cell surface is impaired in PC-3 cells.

Degradation of Intracellular Connexins Via Proteosomal and Lysosomal Pathways

Previous studies have shown degradation of Cxs by both proteosomal and lysosomal pathways (37, 45). Therefore, we next investigated if intracellular accumulation Cx43 and Cx32 was due to their resistance to degradation via proteosomal and lysosomal pathways. Figure 8 shows that treatment with leupeptin, an inhibitor of lysosomal function, and ALLN, an inhibitor of proteosomal pathway (45,46), further increased intracellular accumulation of Cx32 and Cx43 and their steady state level as judged by western blot analysis (Figure 8 B, C). Similar data were obtained with Lactacystin, which is a more specific inhibitor of lysosomal pathway (46). Although we did not measure the half life of intracellular Cx32 and Cx43 in PC-3 cells by pulse-chase analysis, our data suggest that intracellular Cxs are constantly degraded by lysosomal and proteosomal pathway regardless of whether or not they traffic to the cell surface.

Trafficking and Assembly of Connexins into Gap Junctions Induced by α-Catenin

The TX100-solubility, poor cell surface biotinylation, and targeted degradation via proteosomal and lysosomal pathways of Cxs suggested their impaired trafficking to the cell surface as a major cause for intracellular accumulation and ruled out ectopic formation of gap junctions in the intracellular stores or inefficient degradation of Cxs as alternative causes. Because cadherin-mediated adhesion may be nonfunctional in a variety of prostate cancer cell lines (47), including PC-

3, due to deletion of the α-catenin gene (7), we reasoned if cell-cell adhesion conducive for the formation of gap junctions will trigger the trafficking of Cxs from the intracellular stores to the cell surface. Chicken α-catenin, which shows 90 % homology to human α-catenin (**reference**), was introduced transiently into Cx-expressing PC-3 clones using lipofectin. Alternatively, yellow fluorescent protein chimeras of Cx43 and Cx32 were introduced into wild type PC-3 cells simultaneously with chicken α-catenin. Because of the possibility of the regulatory interplay between various junctional complexes and the associated adhesion molecules (1-5), we also studied the expression of E-and N-cadherin after transient α-catenin expression. The data show that α-catenin expression not only triggered the trafficking of Cxs from the intracellular stores to the cell surface but also induced their assembly into gap junctions as judged by immunocytochemical analysis (Figure 9 A) and by TX100-insolubility assay of Cx32 (Figure 9 C) and Cx43 (Figure 9 D). Our data also show that PC-3 cells expressed N- (and not E-) cadherin and its expression did not seem to change after transient expression of α-catenin (Figure 9 B). Furthermore, we found that transient expression of α-catenin also increased the expression of ZO-1 at the areas of cell-cell contact (Figure 9 B).

Intracellular Accumulation of Connexins is a Common Feature of Androgen-independent Prostate Cancer Cell Lines

We next investigated whether impaired trafficking of Cxs from intracellular stores to the cell surface is also observed in other androgen-independent human PCA cell lines. Chimeras of Cx32 and Cx43 fused to yellow fluorescent proteins were introduced transiently into several androgen-independent PCA cell lines and as a positive control into Cx-lacking wild-type androgen-dependent LNCaP cells (Figure 10, and Table 2). The site of localization of these chimeras as well as formation of gap junctions was examined at 24 and 48 h post-transfection. Figure 10 shows that transient transfection of Cx32-YFP and Cx43-YFP into ALVA-31, an androgen-independent PCA cell line, results in intracellular localization, whereas that into LNCaP cells in gap junction formation (indicated by arrows in bottom row). The data from several such experiments are summarized in Table 2 (see legends) and corroborate those of Figure 10.

Discussion

The study reported here was motivated by our two sets of previous findings. First, we had observed that epithelial cells in well-differentiated prostate tumors assembled Cxs into gap junctions whereas those in invasive and poorly differentiated prostate tumors did not and, instead, contained Cxs that were localized intracellularly. Second, we had shown that the forced expression of Cx32

and Cx43 — the 2 Cxs expressed by the well-differentiated epithelial cells of the normal prostate — into an indolent and androgen-dependent but Cx-deficient PCA cell line, LNCaP, inhibited growth, induced differentiation, and retarded tumorigenicity (20). The main findings of our present study are that in contrast to indolent LNCaP cells, forced expression of Cx43 and Cx32 into an invasive cell line PC-3, and several other androgen-independent PCA cell lines, resulted in intracellular accumulation in the medial- and trans-Golgi compartment. The accumulation of Cxs was not caused by the formation of ectopic gap junctions in the intracellular compartments or their aggregation into plaques resistant to degradation via proteosomal and lysosomal pathways but by impaired trafficking to the cell surface. Most significantly, transient expression of α-catenin — a cadherin associated protein that links cadherins to the cytoskeleton elements — induced trafficking of Cx32 and Cx43 from medial- and trans-Golgi compartments to the cell surface and their subsequent assembly into gap junctions.

In contrast to indolent, androgen-dependent cell line LNCaP, we failed to detect significant amount of Cx32 and Cx43 in invasive, androgen-independent PC-3 cells upon cell surface biotinylation, which suggests that intracellular accumulation was caused by impaired or inefficient trafficking and not by endocytosis and internalization upon cell surface arrival. Moreover, we adduce the following evidence to substantiate that intracellular accumulation was not caused by an artefact of overexpression of Cx32 and Cx43, formation of annular and ectopic gap junctions (49-52), and mutations in the Cxs that might have impeded trafficking (53-56) but by the pathological state of

PC-3 cells similar to what we observed with the epithelial cells of prostate tumors in vivo (20). First, transient transfection of α-catenin abrogated impaired trafficking of Cx32 and Cx43 and induced their assembly into gap junctions (Figure 9). Second, no intracellular accumulation of Cx32 and Cx43 was observed in indolent, androgen-dependent LNCaP cells which expressed more Cxs than invasive, androgen-independent PC-3 cells when equal amount of total protein was analyzed by western blot analysis (Figure 3). Third, a major fraction of intracellularly accumulated Cx43 and Cx32 in PC-3 cells was TX100-soluble (not assembled into gap junctions), and insoluble fraction was not detected significantly, consistent with the lack of formation of gap junctions. Fourth, intracellular Cx32 and Cx43 continued to be targeted to degradation via proteosomal and lysosomal pathways, indicating that they had not aggregated into degradation-resistant plaques. Fifth, trafficking of Cx32 and Cx43 from ER to Golgi occurred normally in PC-3 cells as evidenced by the colocalization of Cxs with a medial- and trans-Golgi resident protein1, 4 β-galactosyl transferase and poor localization with ER-resident protein calreticulin as well as the effect of inhibitors of protein trafficking (see Figures 5 and 6).

The intrinsic and extrinsic determinants crucial for regulating the degradation, trafficking and assembly of Cxs into gap junctions are poorly understood (37, 45, 57-59). Impaired trafficking of wild-type Cx43 and Cx32, leading to their intracellular accumulation, in PC-3 cells and its abrogation by α-catenin raises several intriguing questions about the molecular mechanisms involved in the trafficking of wild type Cxs and their assembly into gap junctions. It has been proposed that

bi-directional signaling between cell adhesion molecules and Cxs may be important in initiating the formation of gap junctions (34,35, 60). Consistent with this notion, several studies have shown that restoration of cadherin-based cell-cell adhesion induces the assembly of Cxs into gap junctions (58), and conversely, its abolition impedes that assembly (61,62). Because previous studies showed that transient expression of α-catenin in PC-3 cells triggered the recruitment of E-cadherin from the cytoplasm to the cell surface and restored cell-cell adhesion (7), we reasoned that intracellular accumulation of Cx32 and Cx43 might be caused by deficient E-cadherin mediated cell-cell adhesion. Therefore, we expressed α-catenin in PC-3 cells. We chose to express α-catenin transiently because previous attempts to express it stably in PC-3 cells had failed (Wheelock and Johnson, Unpublished). Although our data document unambiguously that transient expression of α-catenin triggered trafficking of both Cx32 and Cx43 and induced gap junction formation, the mechanism involved is likely to be complex and restoration of E-cadherin based cell-cell adhesion alone may not be the cause.

First, in agreement with other studies, we found that both the parental and Cx-expressing PC-3 cells expressed N-, and not, E-cadherin at the cell-cell contact, which appears to be the main cadherin that mediates cell-cell adhesion in PC-3 cells (7,63,64). Moreover, N-cadherin level did not change significantly upon transient expression of α-catenin as assessed immunocytochemically (Figure 9) and by western blot analysis (data not shown). The reason(s) for the discrepancy between our and previous data regarding E-cadherin expression in PC-3 cells are not understood. Second, we

also found that α-catenin expression not only triggered the trafficking and assembly of Cxs into gap junctions but also recruited ZO-1, a tight junction and adherens junction associated protein, to the cell surface. Third, ZO-1 and Cx32 and Cx43 were not only co-localized with α-catenin at the areas of cell-cell contact but also in the cytoplasm (data not shown). Thus, it is possible that the trafficking of both Cx32 and Cx43 from intracellular stores to the cell surface as well as their assembly into gap junctions are mediated via direct or indirect interaction with ZO-1 — a scaffolding protein with multiple PDZ binding domains which not only has been postulated to play a role in the assembly of gap junctions (65,66) but also of other cell junctions such as tight junctions (67,68). Fourth, the trafficking of Cx32 and Cx43 from intracellular stores to the cell surface as well as formation of gap junctions could be induced significantly upon increasing intracellular cAMP level (unpublished results), suggesting that alternative pathway(s), independent of α-catenin and cadherin-mediated cell-cell adhesion exist.

The molecular mechanisms by which cadherins and their associated proteins may facilitate the assembly of Cxs into gap junctions are likely to be complex in the light of the diversity of the cadherin super-family, dynamic nature of cell-cell adhesion and the diverse nature of its regulation by extraneous and cytoplasmic effectors (1,2,67-71). For example, α-catenin has been shown to interact with vinculin, ZO-1, α-actinin, and actin besides binding to E-cadherin and has also been proposed to be one of the key regulators of the structural integrity of several junctional complexes (67-69). Previous studies, which implicated the role of cadherin-mediated cell-cell adhesion in

facilitating the assembly of Cxs into gap junctions, utilized cell lines in which Cxs trafficked normally, and did not accumulate intracellularly, and in which only the capacity to assemble Cxs into gap junctions was defective (68, 72). Also, in several other studies intracellular accumulation of Cxs was ascribed to an artifact of overexpression and/or internalization and ectopic formation of gap junctions (49,50). Therefore, the role of cell-cell adhesion in enhancing the formation of gap junctions indirectly via its effect on trafficking could have been overlooked. Moreover, the role of adhesion in facilitating the formation of gap junctions is further complicated by other studies showing inhibition of gap junction formation upon restoration of cell-cell adhesion mediated by N-cadherin (73) or vice versa (62, 74). Our data clearly show that N-cadherin is expressed significantly at the cell-cell contact areas (Figure 9 B). Because the weak adhesive force of the extracellular domain of N-cadherin is sufficient for increasing cell-cell adhesion in many cell model systems and α-catenin has been shown to control the strength of adhesion through its interaction with α-actinin and cytoskeleton (67), it is possible that α-catenin induces the trafficking of Cxs and their assembly into gap junctions by modulating the strength of cell-cell adhesion. Because constitutive expression of α-catenin in PC-3 cells is lethal, we could not rule out this possibility.

It is possible that α-catenin may induce the trafficking of Cxs and their assembly into gap junctions by activating multiple pathways and cell-cell adhesion may be just only one such pathway (75). For example, conditional ablation of α-catenin in keratinocytes has been shown to increase their proliferation by activating mitogen-activated protein kinase independent of effect on cell-cell

adhesion (76). Several signal transduction pathways have been shown to regulate the formation and dissolution of gap junctions (13-19). It is it is possible that α-catenin triggered the trafficking of Cx32 and Cx43 by activating multiple pathways. In this regard we note that mitogen-activated protein kinase has been shown to be constitutively activated not only in PC-3 cells but also in several other androgen-independent prostate cancer cell lines in which α-catenin has been deleted (77).

Impaired trafficking of Cxs has previously been observed in a number of other diseases — such as X-linked Charcot- Marie- Tooth disease, sensorineural hearing loss, erythrokeratoderma variabilis, visceroatrial heterotaxy and cataract — but the impairment in most cases has been causally linked to the mutations in the Cxs themselves and not to the pathological state of the cells (54-56). Our data have documented impaired trafficking as an additional cause of communication deficiency in tumor cells and support the notion that the control of transport of Cxs from intracellular stores to cell surface by physiological effectors could yet be another way to control the formation and dissolution of gap junctions as has been recently shown (74). While this work was in progress, Hernandez-Blazquez et al. have also shown that transport of Cxs from the ER to the cell surface is controlled by E-cadherin (78). (Needs additional touch).

The incidence of PCA increases with age and is characterized by progression from an indolent, slow-growing androgen-dependent state to an invasive, androgen-independent state (79,80, 81). Because intracellular accumulation of Cx43 and Cx32 was observed only in epithelial cells from

invasive prostate carcinomas (20) and in androgen-independent cell lines (present study), but not in an indolent and androgen-dependent cell line LNCaP (20), it is tempting to speculate that the pathways governing the trafficking of Cxs and their subsequent assembly into gap junctions become altered during the progression of PCA from androgen-dependent to independent state. Our Cx43- and Cx32-expressing androgen-independent PC-3 cells in which α-catenin is deleted, and androgen-dependent LNCaP cells in which E-cadherin-based adhesion system has remained intact (20), offer 2 in vitro systems that mimic the behavior of Cxs in vivo and should prove useful in elucidating the molecular mechanisms by which α-catenin controls the trafficking of Cx43 and Cx32 and their assembly into gap junctions.

Acknowlegments

This research was supported by an NIH grant CA73769 and DOD grant (DAMD-17-00-1-0032). We thank Birgit Rose, Bernard A. Roos and Carlos Perez-Stable for encouragement and friendship.

References

1. Geiger B, Bershadzky A, Pankov R, and Yamada KM (2001) *Nature Rev Cell Mol Biol* **2,** 793-805

2. Yap AS, Brieher WM, and Gumbiner BM (1997) *Ann Rev Cell Dev Biol* **13,** 119-146

3. Aberle H, Schwartz H, and Kemler R (1996) *J Cell Biochem* **61,** 514-523

4. Stevenson BR and Keon BH (1998) *Ann Rev Cell Dev Biol* **14,** 89-109

5. Garrod D, Chidgey M, and North A (1996) *Curr Opin Cell Biol* **8,** 670-678

6. Morton RA, Ewing CM, Nagafuchi A, Tsukita S, and Isaacs WB (1993) *Cancer Res* **53,** 3585-3590

7. Ewing CM, Ru N, Morton RA, Robinson JC, Wheelock MJ, Johnson KR, Barrett JC, and Isaacs WB (1995) *Cancer Res* **55,** 4813-4817

8. Jarrad DF, Paul R, Bokhoven AV, Nguyen SH, Bova GS, Wheelock MJ, Johnson KR, Schalken J, Bussemakers M, and Isaacs WB (97 A.D.) *Clin Cancer Res* **3,** 2121-2128

9. Kleinerman DI, Troncoso P, Lin S-W, Pisters LL, Sherwood ER, Brooks T, Eschenbach ACV, and Hsieh J-T (1995) *Cancer Res* **55,** 1215-1220

10. Isaacs JT (1997) *Am J Pathol* **150,** 1511-1521

11. Isaacs JT (1999) *Hormone refractory prostate cancer* **26,** 263-273

12. Hsieh J-T, Luo W, Song W, Wang Y, Kienerman DI, Van NT, and Lin SH (1995) *Cancer Research* **55,** 190-197

13. Loewenstein WR and Rose BR (1992) *Semi Cell Biol* **3,** 59-79

14. Bruzzone R, White TW, and Paul DL (1996) *Eur J Biochem* **238,** 1-27

15. Goodenough DA, Goliger JA, and Paul DA (1996) *Ann Rev Biochem* **65,** 475-502

16. Kumar NM and Gilula NB (1996) *Cell* **84,** 381-388

17. Trosko JE and Ruch RJ (1998) *Fronties Biosci* **3,** 208-236

18. Yamasaki H and Naus CGS (1996) *Carcinogen* **17,** 1199-1213

19. Ruch RJ (1994) *Ann Clin Lab Sci* **24,** 216-231

20. Mehta PP, Perez-Stable C, Nadji Mehrdad, Mian M, Asotra K, and Roos BA (1999) *Dev Genet* **24,** 91-110

21. Zhu D, Caveney S, Kidder GM, and Naus CCG (1991) *Proc Natl Acad Sci, USA* **88,** 1883-1887

22. Chen S-C, Pelletier DB, Ao P, and Boynton AL (1995) *Cell Growth Diff* **6,** 681-690

23. Mesnil M, Krutovskikh V, Piccoli C, Elfgang C, Traub O, Willecke K, and Yamasaki H (1995) *Cancer Res* **55,** 629-639

24. Statuto M, Audebet C, Tonoli H, Selmi-ruby S, Rousset B, and Munari-Silem Y (1997) *J Biol Chem* **272,** 24710-24716

25. Temme A, Buchmann A, Gabriel H-D, Nelles E, Schwarz M, and Willecke K (1997) *Curr Biol* **7,** 713-716

26. Abate-Shen C and Shen MM (2000) *Genes &Development* **14,** 2410-2434

27. Mehta PP, Lokeshwar BL, Schiller PC, Bendix MV, Ostenson RC, Howard GA, and Roos BA

(1996) *Mol Carcinogen* **15,** 18-32

28. Mehta PP, Hotz-Wagenblatt A, Rose B, Shalloway D, and Loewenstein WR (1991) *J Membr Biol* **124,** 207-225

29. Mehta PP, Yamamoto M, and Rose B (1992) *Mol Biol Cell* **3,** 839-850

30. Nagy JI, Li WE, Roy C, Doble BW, Gilchrist JS, Kardami E, and Hertzberg EL (1997) *Exp Cell Res* **236,** 127-136

31. Goodenough DA, Paul DL, and Jesaitis L (1988) *J Cell Biol* **107,** 1817-1824

32. Kim J-B, Islam S, Kim YJ, Prudoff RS, Sass KM, Wheelock MJ, and Johnson KR (2000) *J Cell Biol* **151,** 1193-1205

33. Miller AD and Rosman GJ (1989) *Biotechniques* **7,** 980-990

34. Musil LS and Goodenough DA (1991) *J Cell Biol* **115,** 1357-1374

35. Musil LS and Goodenough DA (1993) *Cell* **74,** 1065-1077

36. VanSlyke JK and Musil LS (2000) *Methods* **20,** 156-164

37. VanSlyke JK, Deschenes SM, and Musil LS (2001) *Mol Biol Cell* **11,** 1933-1946

38. Mehta PP, Bertram JS, and Loewenstein WR (1986) *Cell* **44,** 187-196

39. El-Fouly MH, Trosko JE, and Chang C-C (1987) *Exp Cell Res* **168,** 422-430

40. Johnson S, Michalak M, Opas M, and Eggleton P (2001) *Trends Cell Biol* **11,** 122-129

41. Yamaguchi N and Fukuda MN (1995) *J Biol Chem* **270,** 12170-12176

42. Klausner RD, Donaldson JG, and Lippincott-Schwartz J (1992) *J Cell Biol* **116,** 1071-1080

43. Tartakoff AM (1983) *Cell* **32,** 1026-1028

44. Thyberg J and Moskalewski S (1999) *Exp Cell Res* **246,** 263-279

45. Berthoud VM, Tadros PN, and Beyer EC (2001) *Methods* **20,** 180-187

46. Lee HD and Goldberg AL (1998) *Trends Cell Biol* **8,** 397-403

47. Umbas R, Isaacs WB, Bringuier PP, Xue Y, Debruyne FMJ, and Schalken JA (1997) *Int J Cancer* **74,** 374-377

48. Johnson KR (2002) *J Cell Biol*

49. Naus CCG, Hearn S, Zhu D, Nicholson BJ, and Shivers RR (1993) *Exp Cell Res* **206,** 72-84

50. Kumar NM, Friend DS, and Gilula NB (1995) *J Cell Sci* **108,** 3725-3734

51. Jordon K, Solan JL, Dominguez M, Sia MA, Hand A, Lampe PD, and Laird DW (1999) *Mol Biol Cell* **10,** 2033-2050

52. Jordon K, Chodock R, Hand AR, and Laird DW (2001) *J Cell Sci* **114,** 763-773

53. Deschenes SM, Walcott JL, Wexler TL, Scherer SS, and Fischbeck KH (1997) *J Neuro Sci* **17,** 9077-9084

54. Krutovskikh V and Yamasaki H (2000) *Mutation Res* **462,** 197-207

55. Kelsell DP, Dunlop J, and Hodgins MB (2001) *Trends Cell Biol* **11,** 2-6

56. Kelsell DP, Di W-L, and Houseman MJ (2001) *Am J Hum Genet* **68,** 559-568

57. Martin PEM, Steggles J, Wilson C, Ahmad S, and Evans WH (2000) *Biochem J* **349,** 281-287

58. Martin PEM, Brundell G, Ahmad S, Errington RJ, and Evans WH (2001) *J Cell Sci* **114,** 3845-3855

59. Laird DW, Castillo M, and Kasprzak L (1995) *J Cell Biol* **131,** 1193-1203

60. Musil LS, Cunningham BA, Edelman GM, and Goodenough DA (1990) *J Cell Biol* **111,** 2077-2088

61. Meyer RA, Laird DW, Revel J-P, and Johnson RG (1992) *J Cell Biol* **119,** 179-189

62. Frenzel EM and Johnson RG (1996) *Develop Biol* **179,** 1-16

63. Tomita K, vanBokhoven A, and Jansen JFC (2000) *Biochem Biophys Res Comm* **267,** 870-874

64. Tran NL, Nagle RB, Cress AE, and Heimark RL (1999) *Am J Pathol* **155,** 787-798

65. Toyofuku T, Yabuki M, Otsu K, Kuzuya T, Horrobin DF, and Tada M (1998) *J Biol Chem* **273,** 12725-12731

66. Toyofuku T, Akamatsu Y, Zhang H, Kuzuya T, Tada M, and Hori M (2001) *J Cell Biol* **276,** 1780-1788

67. Provost E and Rimm DL (1999) *Curr Opin Cell Biol* **11,** 567-572

68. Tsukita S, Furuse M, and Itoh M (1999) *Curr Opin Cell Biol* **11,** 628-633

69. Gumbiner BM (2000) *J Cell Biol* **148,** 399-404

70. Barth AIM, Nathke IS, and Nelson WJ (1997) *Curr Biol* **9,** 683-690

71. Fukata M and Kaibuchi K (2001) *Nature Rev Cell Mol Biol* **2,** 887-897

72. Jongen WMF, Fitzgerald DJ, Asamoto M, Piccoli C, Slaga TJ, Gros D, Takeichi M, and Yamasaki H (1991) *J Cell Biol* **114,** 545-555

73. Wang Y and Rose B (1997) *J Cell Sci* **110,** 301-309

74. Paulson AF, Lampe PD, Meyer RA, TenBroek E, Atkinson MM, Walseth TF, and Johnson RG (2000) *J Cell Biol* **113,** 3037-3049

75. Nagafuchi A (2001) *Curr Opin Cell Biol* **13,** 600-603

76. Vasioukhin V, Bauer C, Degenstein L, Wise B, and Fuchs E (2001) *Cell* **104,** 605-617

77. Chen T, Cho RW, Stork PJS, and weber MJ (1999) *Cancer Res* **59,** 213-218

78. Hernandez-Blazquez FJ, Joazeiro PP, Omori Y, and Yamasaki H (2001) *Exp Cell Res* **270,** 235-247

79. Isaacs WB, Bova GS, Morton RA, Bussemakers MJG, and Ewiing CM (1994) *Semi Oncol* **21,** 514-521

80. Klien KA, Reiter RE, Redula J, Moradi H, Zhu XL, Brothman AR, Lamb DJ, Marcelli M, Belldegrun A, Witte ON, and Sawyers CL (1997) *Nature Genet* **3,** 402-408

81. Hegarty NJ, Fitzpatrick MJ, Richie JP, Scardino PT, White RW, Schroder FH, and Coffey DS (1999) *The Prostate* **40,** 261-268

Figure 1. Retroviral mediated transduction and expression of Cx32 and Cx43 in PC-3 cells.

PC-3 cells were infected with the control and Cx43- and Cx32-harboring recombinant retroviruses and the Cx levels were determined in several independent clones by northern (A) and western blot (B,C) analyses of total RNA (20 μg) and protein (15 μg). Note Cx43-expressing PC-3 clones (lanes PC-43-1 to PC-43-4) transcribe the larger (4.4 kb) exogenous retroviral Cx43 mRNA (A, labeled R-Cx43) abundantly and undetectable or a low level of smaller (3 kb) endogenous Cx43 mRNA (labeled E-Cx43). Western blot analysis of total cell lysates probed with the polyclonal anti-Cx43 (B) and a monoclonal anti-Cx32 (C) antibody. PC-WT= parental PC-3 cells; PC-NEO-1 to PC-NEO-4= control PC-3 clones isolated after infection with LXSN; PC-43-1 to PC-43-9= PC-3 clones isolated after infection with LXSN43S; PC-32-1 to PC-32-10= PC-3 clones isolated after infection with Cx32-containing retrovirus; H-PROST= human prostate; M-PROST= mouse prostate; M-HEART= mouse heart. Position of the molecular weight markers is indicated.

Figure 2. Immunolocalization of Cx32 and Cx43 in invasive PC-3 and indolent LNCaP prostate cancer cell lines.

Cells were immunostained with polyclonal anti-Cx43 and monoclonal anti-Cx32 antibodies as described in Methods. Note most immunostaining in invasive PC-3 cells that express Cx43 (row 1) and Cx32 (row 3) is intracellular and only few punctate dots characteristics of gap

junctional plaques are seen at the cell-cell contact areas (row 1, arrows). In indolent LNCaP cells that express Cx43 (row 2) and Cx32 (row 4), no intracellular immunostaining is observed and most punctate dots are localized at the cell-cell contact areas (rows 2 and 4, arrows). Note also that in some cells (yellow arrows) scattered punctate dots are observed throughout the cytoplasm.

Figure 3. Triton X-100-insolubility of Cx32 and Cx43 in invasive PC-3 and indolent LNCaP prostate cancer cell lines.

Triton X 100-soluble and -insoluble extracts from PC-3 and LNCaP cells were analyzed by western blot analysis as described in Methods. Note that only a small fraction of total Cx43 (A) and Cx32 (B) remained in TX100-insoluble form in invasive PC-3 cells whereas a major fraction of total Cx43 and Cx32 remained in TX100-insoluble form in indolent LNCaP cells and in RL-CL9 cells. C. Triton X-100-insolubility of Cx32 and Cx43 in invasive PC-3 and indolent LNCaP prostate cancer cell lines in situ. Invasive PC-3 and indolent LNCaP cells were extracted in situ with 1 % Triton X-100 as described in Methods and immunostained with polyclonal anti-Cx43 and monoclonal anti-Cx32 antibodies. Note that in Cx43- and Cx32-expressing PC-3 cells, nearly all intracellular Cxs are lost upon in situ extraction compared to Cx-expressing LNCaP cells which form abundant gap junctions.

Figure 4. Gap junctional communication in Cx43- and Cx32-expressing PC-3 cells.

Examples of junctional transfer of LY in parental PC-3 cells (PC-3-WT), Cx43-expressing (PC-3-43), and Cx32-expressing PC-3 cells (PC-32) as assayed by scrape-loading (colored rows) and by microinjection of LY (black and white row). Cells were scrape-loaded and microinjected as described (see Methods) and the cell-cell transfer of the fluorescent tracer is shown 5 min after scrape-loading and microinjection. Note gap junctional communication is not significantly enhanced in Cx-expressing PC-3 cells compared to parental cells.

Figure 5. Intracellular Connexin43 and connexin32 in invasive PC-3 cells reside in medial- and trans-Golgi network.

Connexin32- and connexin43-expressing PC-3 clones were immunostained with calreticulin (Labeled CALRET) and anti-Cx32 (Labeled CONNEXIN32) and Cx43 (Labeled CONNEXIN43) antibodies (Top 2 rows). The colocalization of Cxs and calreticulin was studied by z-sections and deconvolution as described in the Methods. Note significant lack of colocalization of Cxs with calreticulin. Connexin32 and connexin43-expressing PC-3 clones were grown on glass cover slips and transiently transfected with plasmid pCFP-Golgi that codes for a cyan fluorescent protein (green in this figure) fused to the N-terminal 81 amino acids of human β-1,4-galactosyl transferase (Labeled β-1,4-GT). Cells were fixed in 2% paraformaldehyde, permeabilized and immunostained with anti- Cx43 and -Cx32 (red) antibodies. The colocalization was studied as described abobe.. Note the extensive overlapping between Cxs and β-1,4-GT.

Figure 6. The effect of brefeldin, monensin and nocodazole on the distribution of Connexin43 and connnexin32 in PC-3 cells.

Connexin43- and connexin32-expressing PC-3 clones were grown on glass cover slips for 3 days and treated with brefeldin A (20 μM, 8h), monensin (10 M, 8h) and nocodazole (20 μM,6h). Cells were then immunostained with anti-Cx43 and -Cx32 antibodies. Note that brefeldin A treatment reduced the intensity of immunostaining and abolished the typical paranuclear immunostaining pattern of Cxs whereas monensin treatment had no effect. Note also distinctly scattered immunostaining pattern throughout the cytoplasm after nocodazole treatment.

Figure 7. Cell surface biotinylation of connexin43 and connexin32 in PC-3 cells.

Confluent connexin32- and connexin43-expressing PC-3 and LNCaP cells were surface biotinylated at 4 ^{0}C for 30 minutes and analyzed by wesstern blot analysis as described in the Methods.

Please check raj ==========Lane 1 and 2 represent the total and biotinylated fractions of Cx43 and Cx32, respectively. Data presented are representative of 3 independent experiments. A & B. Recovery of biotinylated Cx 43/32 from PC-3-43/32 and LNCaP-43/32 cells

Note the lesser amounts of biotinylated fraction of Cx43/32 in PC-3-43 cells when compared to the total fraction. Also Cx43/32 signals in the biotinylated fraction of PC-3-43/32 cells were

evident only after longer exposure of the blots. Note also the equal intensities of Cx-43/32 in total and biotinylated fraction of LNCaP-43 cells.

Figure 8. Intracellularly accumulated connexin43 and connexin32are degraded via proteosomal and lysosomal pathways.

A. Connexin43- and connexin32-expressing PC-3 clones were grown on glass cover slips for 3 days and treated with ALLN (100 μM) and leupeptin (10 μM) for 12 h. Cells were then immunostained with anti-Cx43 and -Cx32 antibodies as described in the Methods. Note an increase in the intensity of intracellular immunostaining after treatment with both ALLN and Leupeptin compared to controls. Similar data were obtained with lactacystin. B. Connexin43- and connexin32-expressing PC-3 clones were grown in 10 cm culture dishes for 3 days and treated with ALLN(100 μM.) and leupeptin (10 μM) for 12 h. Cells. were treated with carrier (lane 1 and 4), 100 _M ALLN, (lane 2 and 5) or 10 _M Leupeptin (lane 3 and 6) for 12 h. The level of connexin43 and connexin32 was determined in cell lysates prepared after treatment of cells with the vehicle (lanes 1,4), ALLN (lanes 2 and 5) and leupeptin (lanes 3 and 6) by western blot analysis as described in the Methods. Note an increase in the level of both connexin32 and connexin43 in ALLN- and Leupeptin-treated cells. β-actin was used as loading control and 10 μg of protein was loaded per lane..

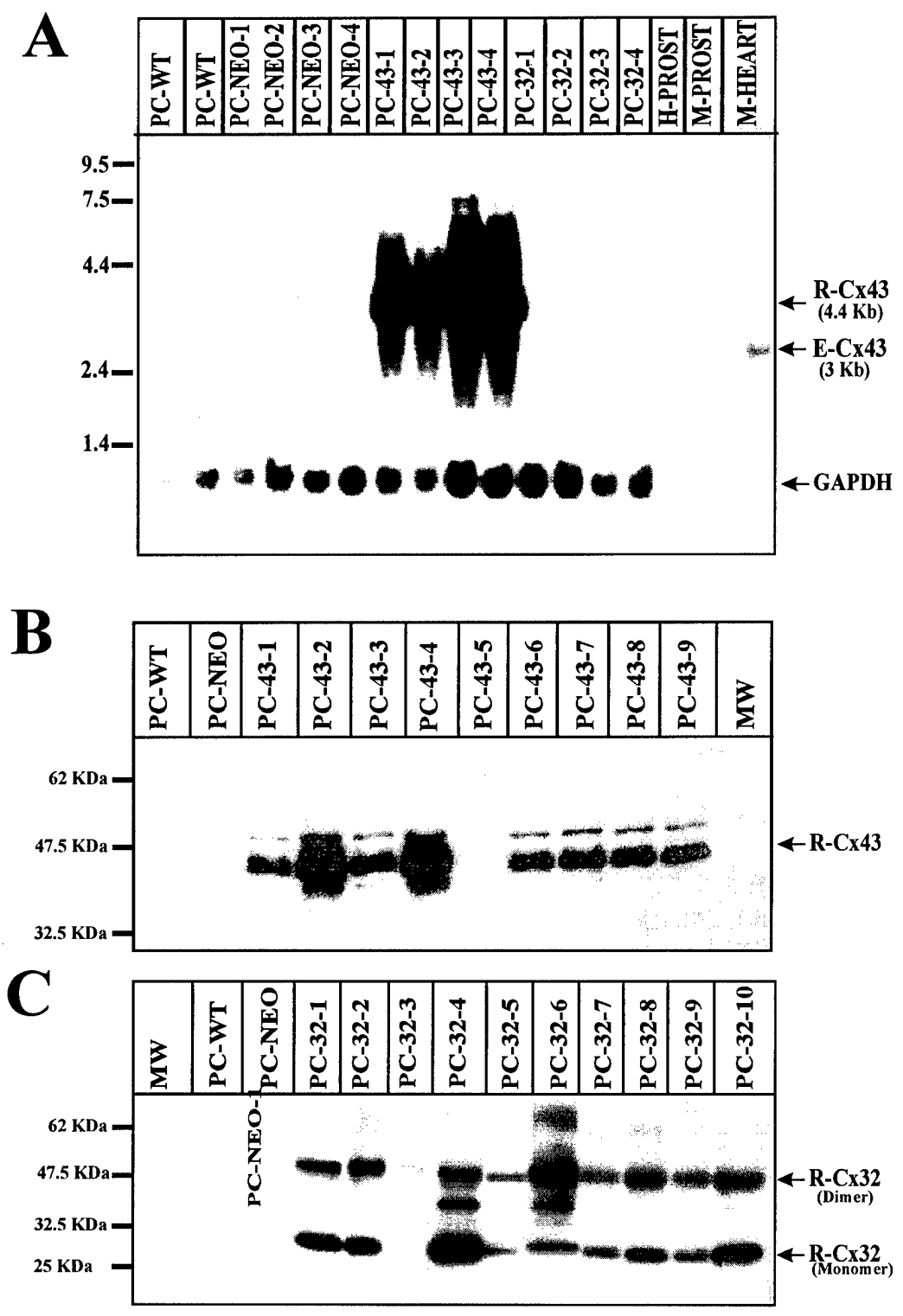
Figure 9. Transient transfection of α-catenin induces trafficking of connexins and formation of gap junctions.

A. Parental PC-3 cells were grown on glass cover slips and transiently transfected with pcDNA3-α-catenin and pCx43-YFP and pCx32-CFP. After 48 h, cells were fixed in 2% paraformaldehyde, permeabilized and immunostained with antibody against α-catenin (red). The colocalization of Cxs (green) with α-catenin was studied after acquiring z-section images and deconvolution as described in the Methods. Note the formation of puncta (gap junctions) at the cell-cell contact areas in cells expressing α-catenin. Areas of cell-cell contact and puncta are indicated by arrows. Last row is higher magnification of row 3. Similar data were obtained with connexin32- and connexin43-expressing PC-3 clones. B. Expression of N-cadherin and ZO-1 in connexin32 and connexin43-expressing PC-3 clones after transient transfection with pcDNA3-α-catenin. Control = untransfected; Transfected = Cells transfected with pcDNA3-α-catenin. Note absence of E-cadherin expression and increased expression of ZO-1 in transfected cells. C, D. Connexin32- and connexin43-expressing PC-3 clones (C, D, respectively) were seeded in 10 cm dishes and allowed to grow to 50 % confluence for 3 days. Cells were then transiently transfected with pcDNA3-α-catenin and the assembly of gap junctions was studied by Triton X-100 insolubility assay 48 h after transfection. Control = Untransfected cells; and Transfected = Cells transfected with pcDNA3-α-

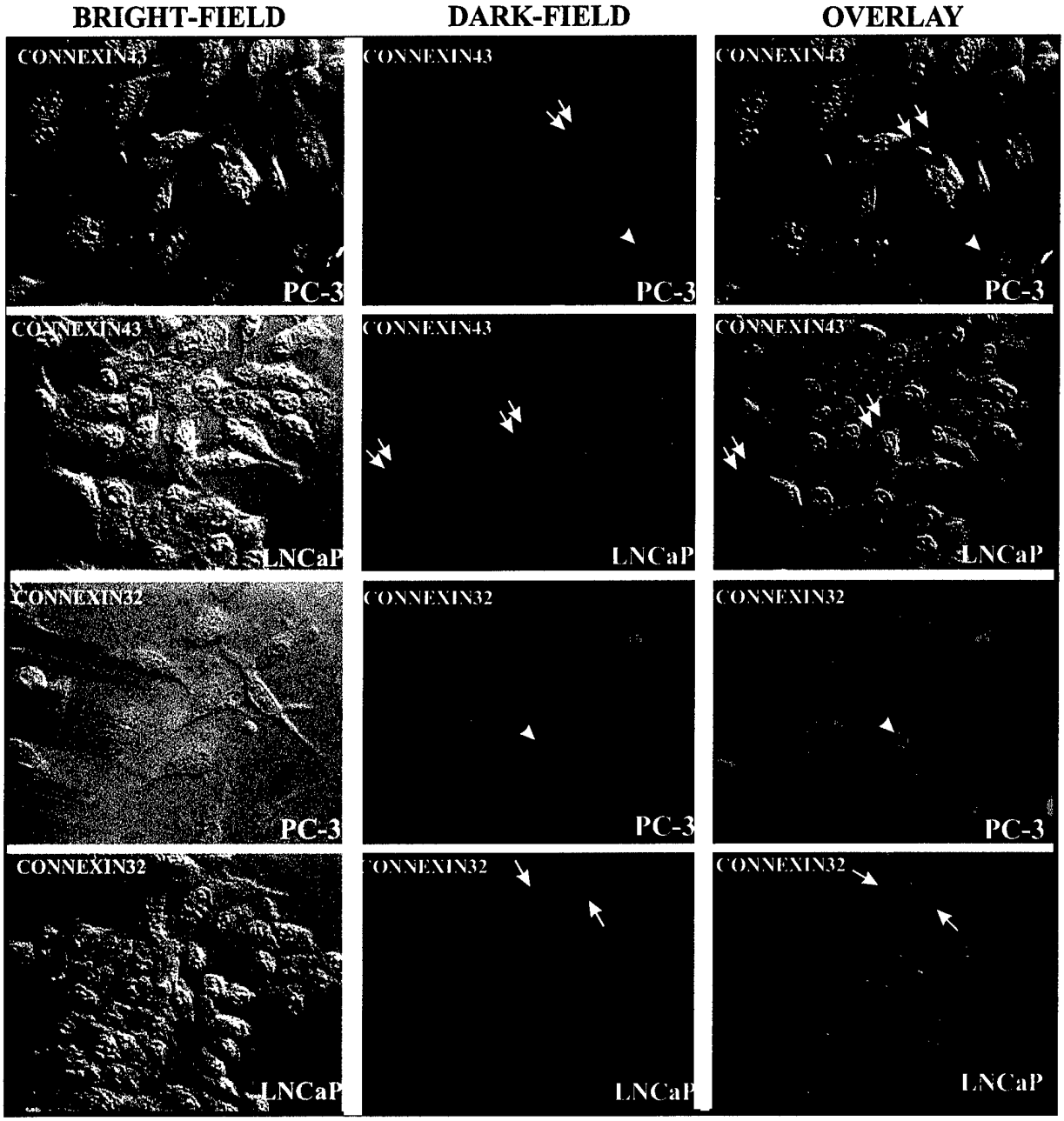
catenin. Note the appearance of Tx100-insoluble bands after in cells transfected with pcDNA3-α-catenin.

Figure 10. Impaired trafficking of connexin43 and connexin32 in other human prostate cancer cell lines.

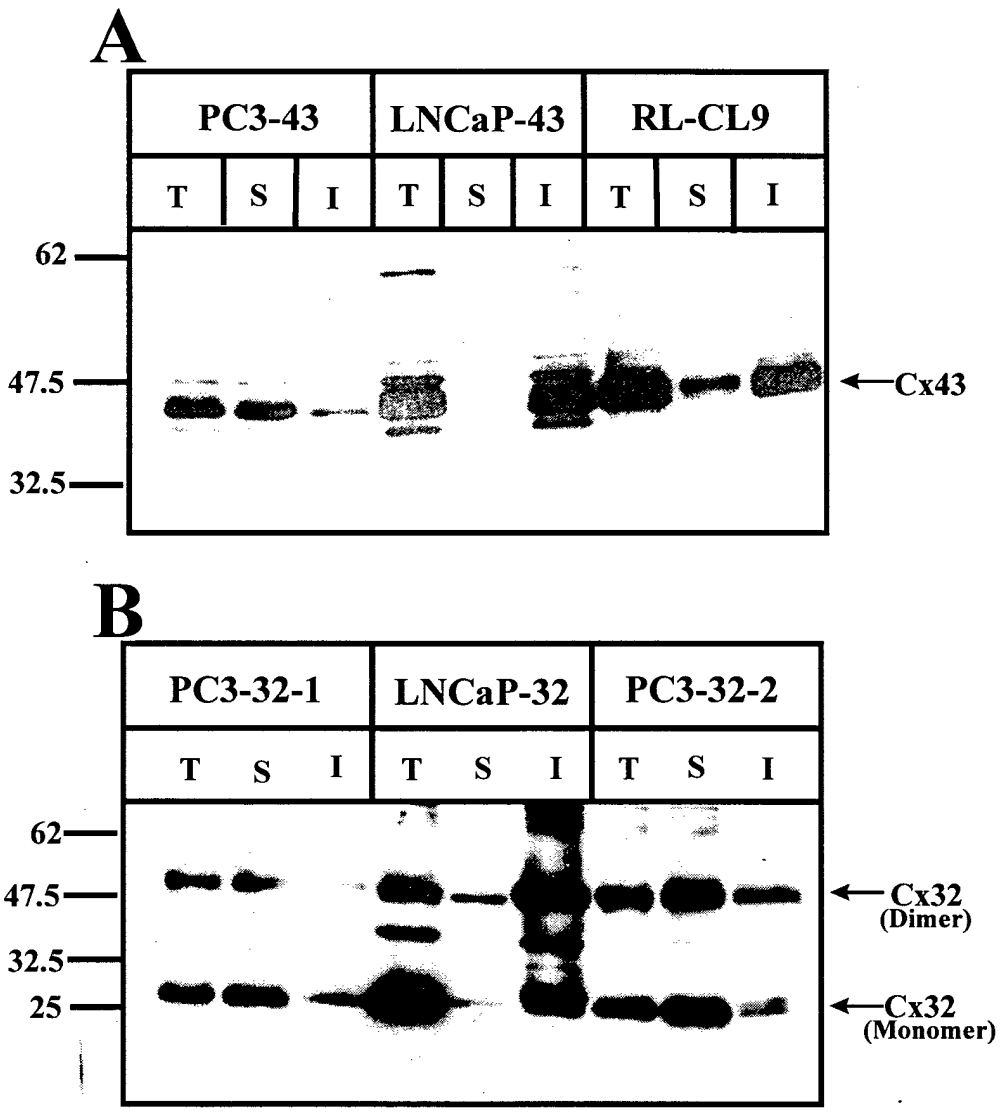
Several androgen-independent human PCA cell lines (see Table 2) were grown on glass cover slips and transiently transfected with a control plasmid pEYFP-N1 (labeled YFP) and pCx43-YFP and pCx32-CFP. Androgen-dependent LNCaP cells were used as a positive control. Cells were fixed in 2% paraformaldehyde 48 h post-transfection and localization of connexin43 and connexin32 in the intracellular stores and at the cell-cell contact areas was observed under fluorescent microscope. Note paranuclear localization of Cx43 and Cx32 chimeras fused to fluorescent protein in androgen independent PCA cell line and formation of gap junctions at the cell-cell contact areas (arrows, bottom row) in androgen-dependent LNCaP cells. Note also the diffuse distribution of enhanced yellow fluorescent protein alone in LNCaP and androgen-independent cells.



Govindarajan et al Figure 1



Govindarajan et al. Figure 2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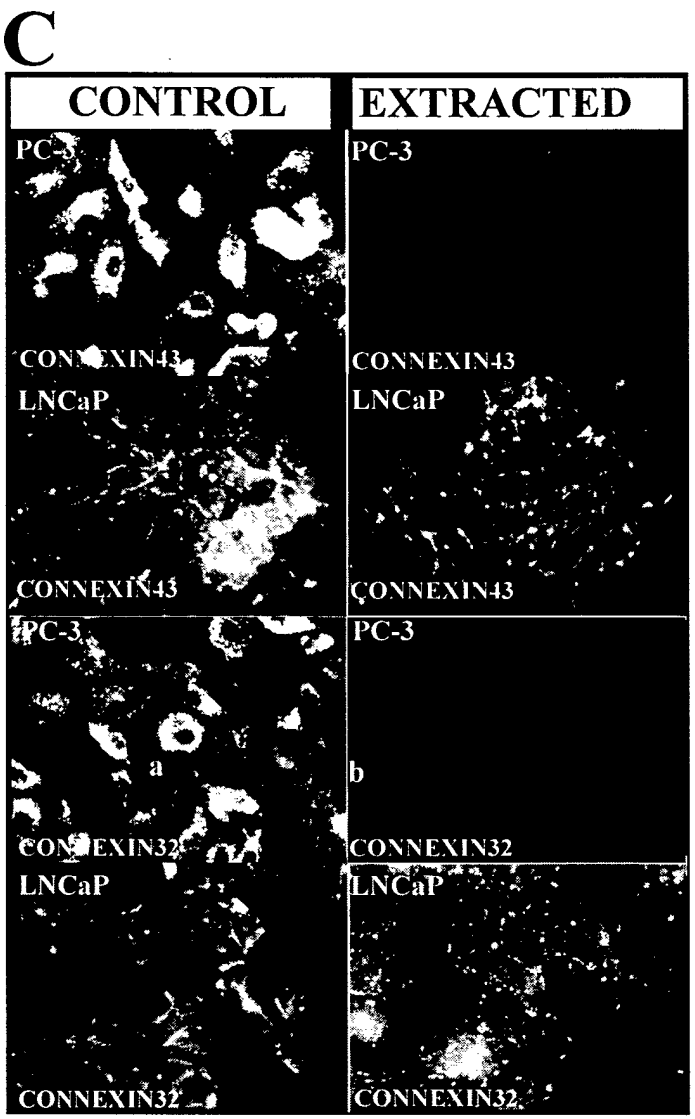
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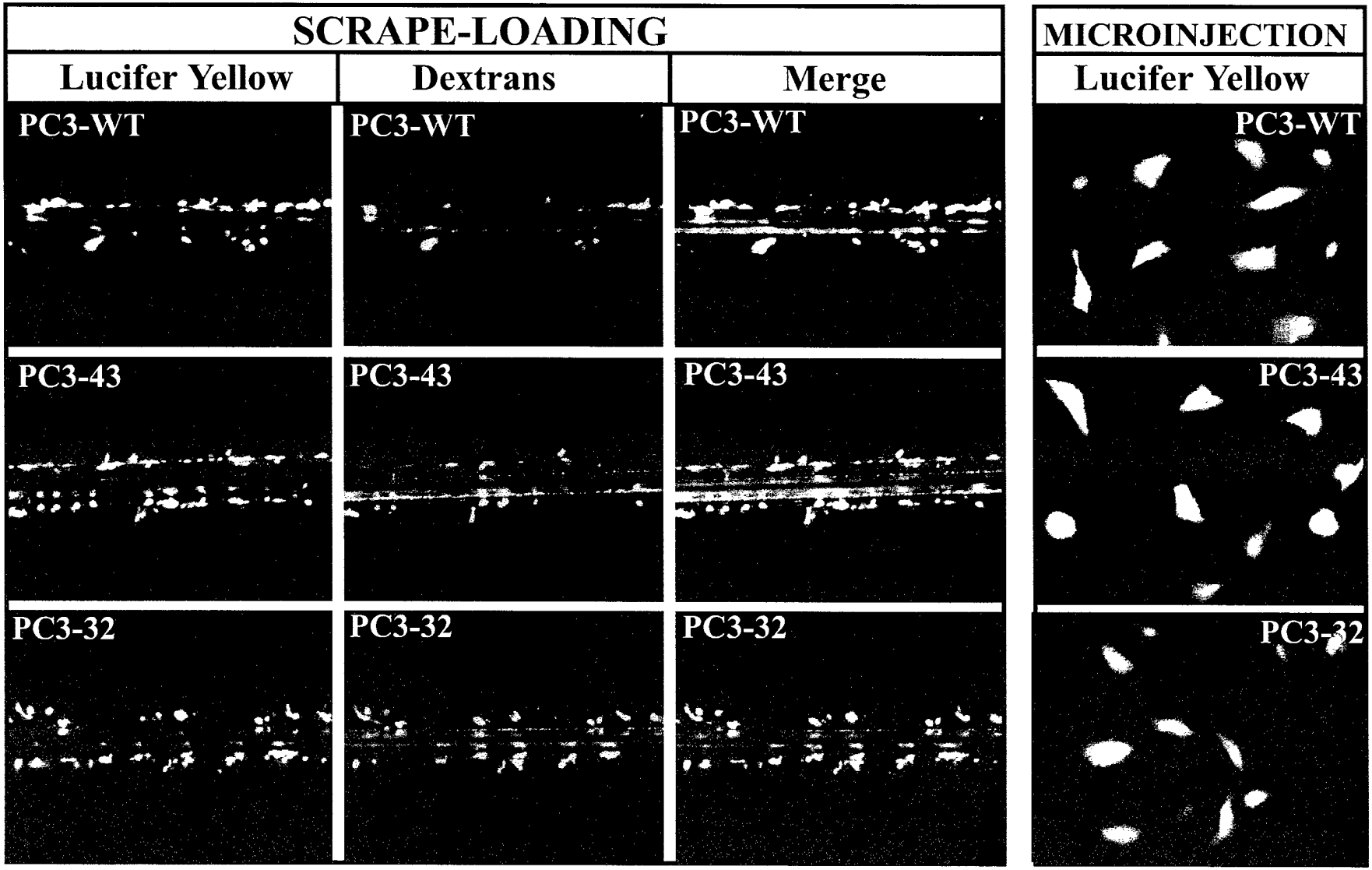
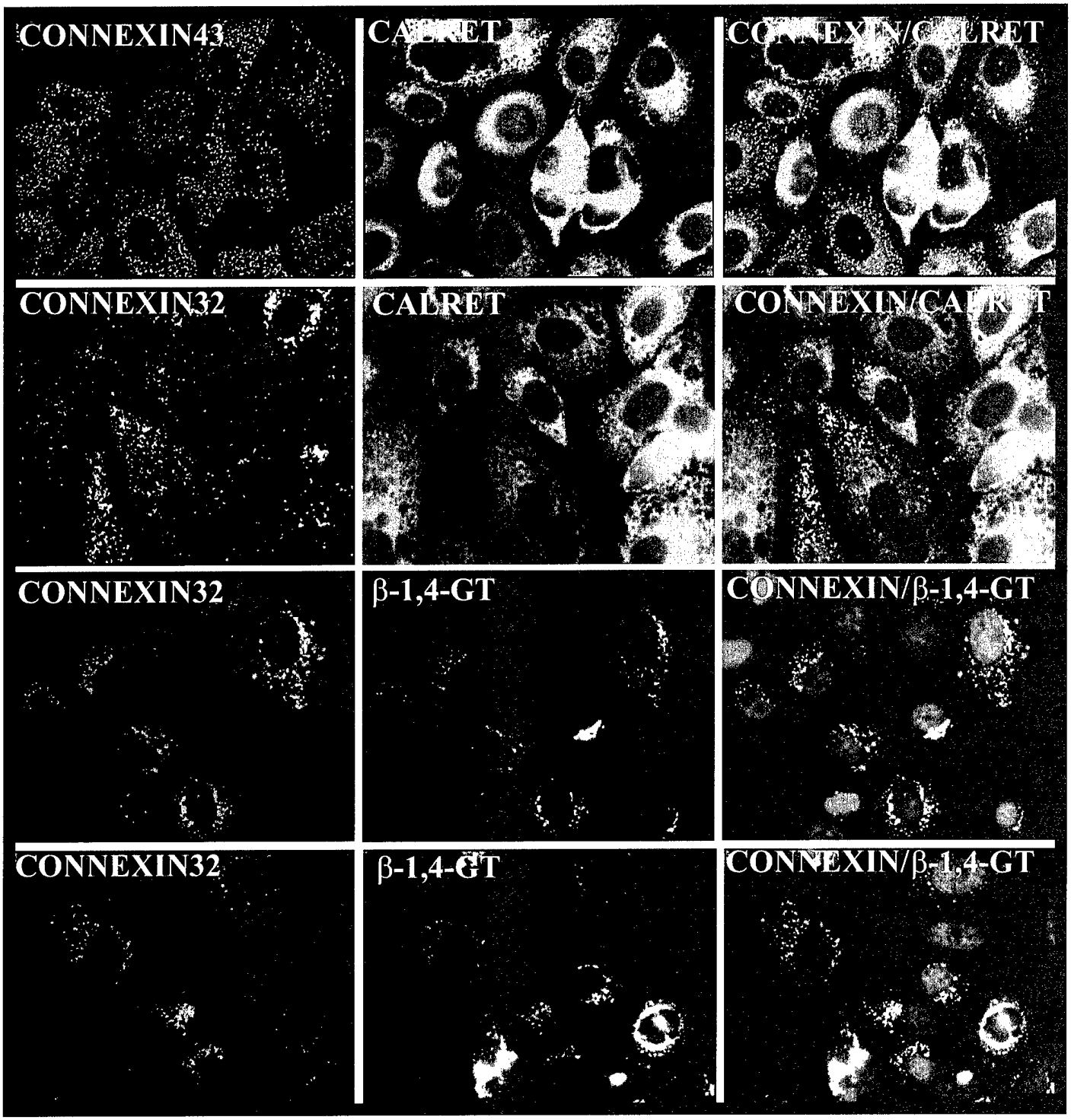
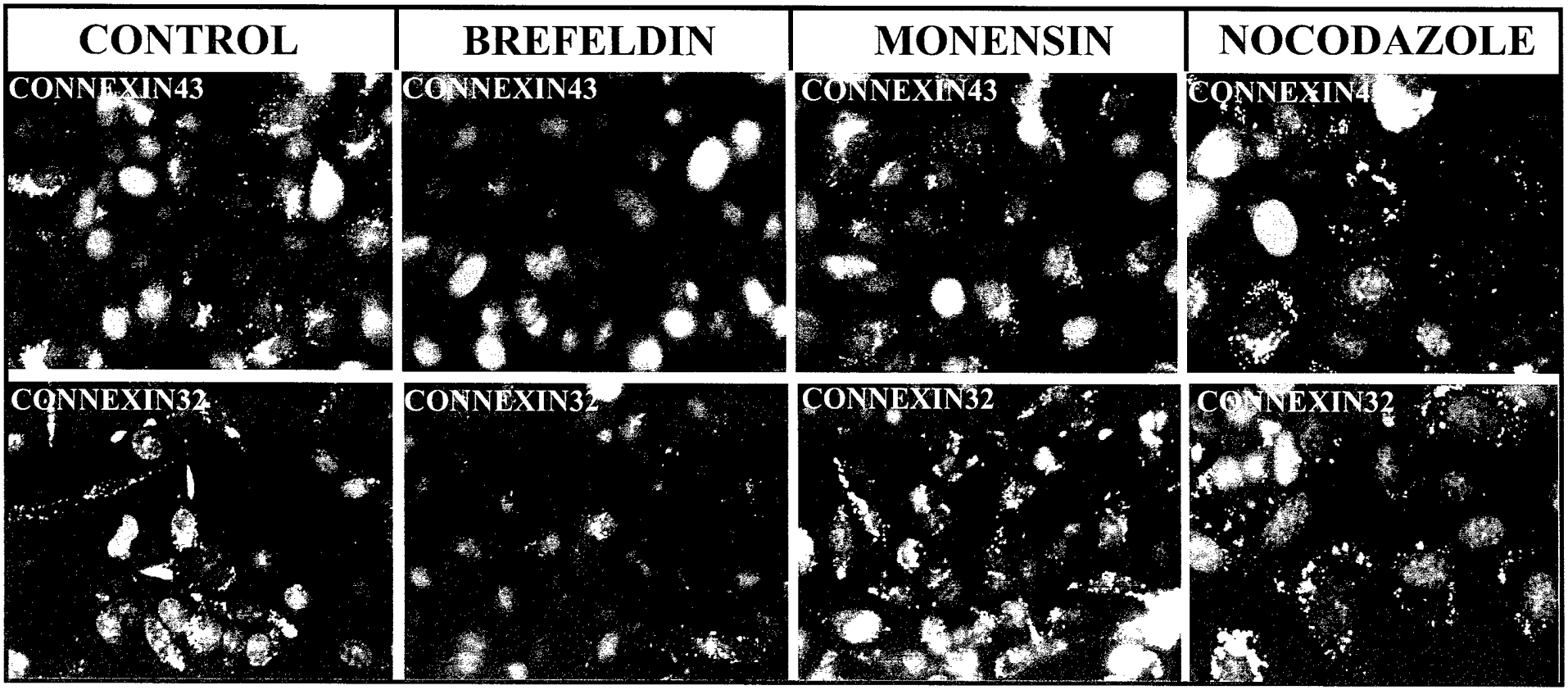


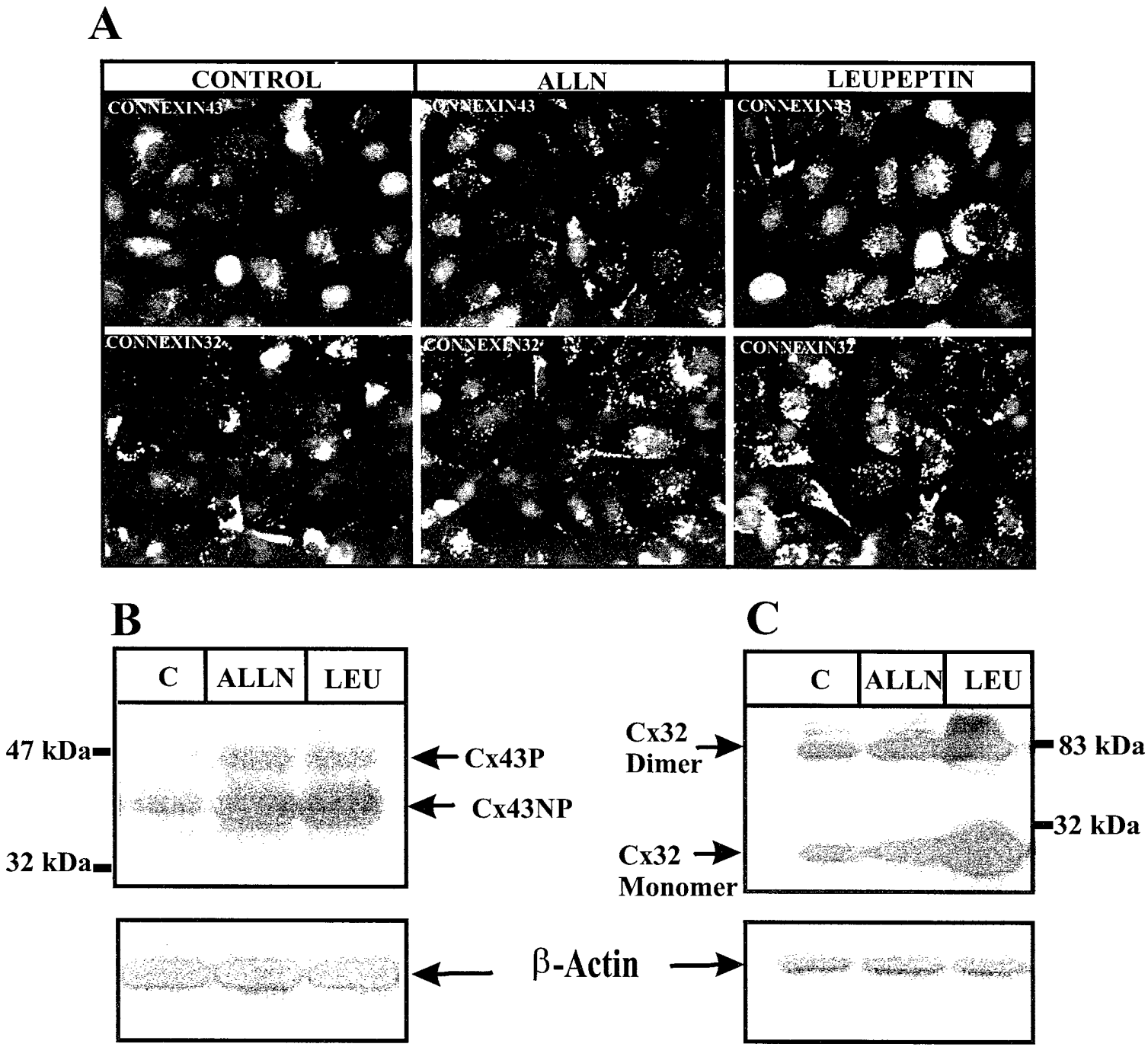
Govindarajan et al. Figure 4



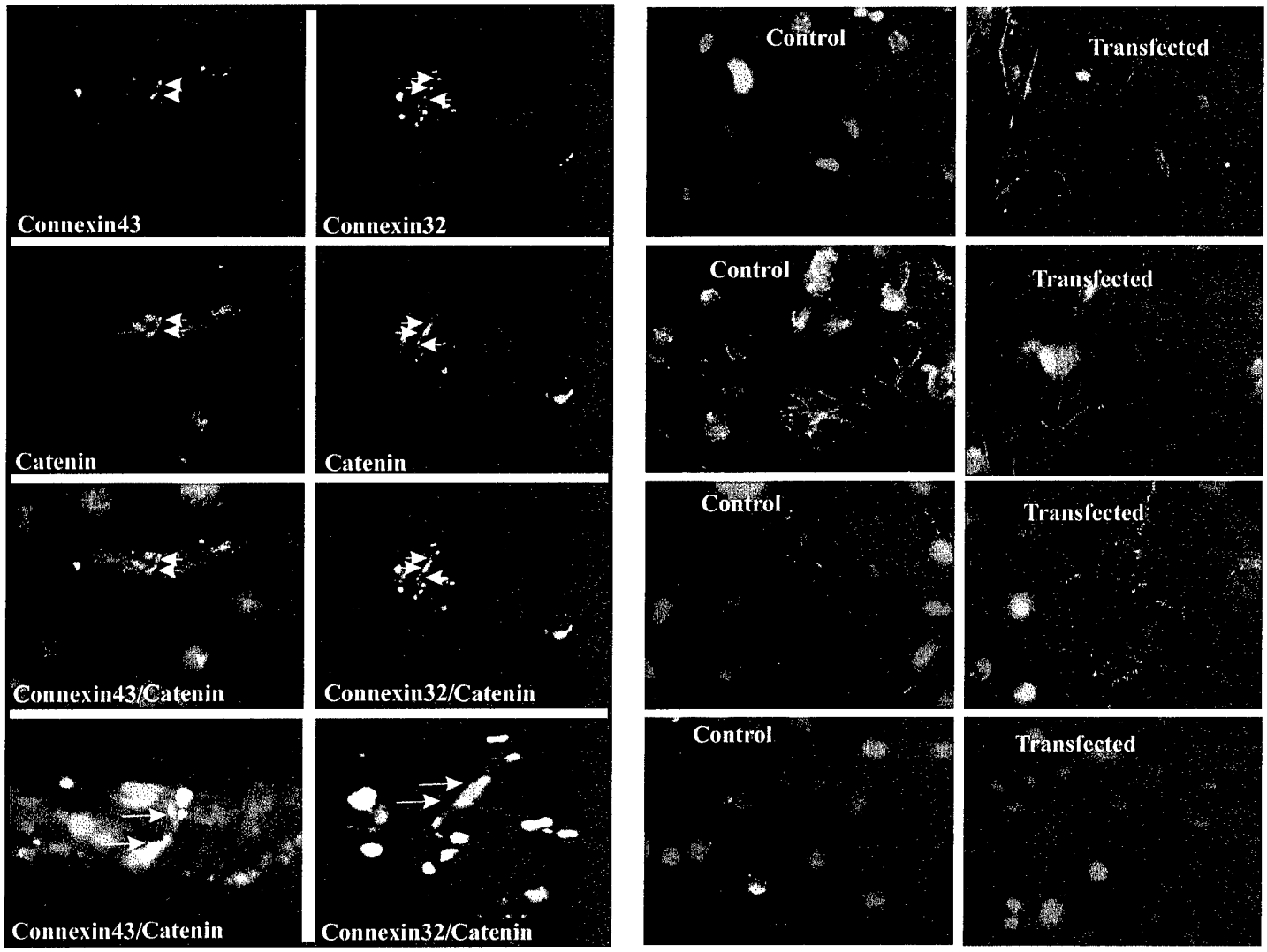
Govindarajan et al. Figure 5

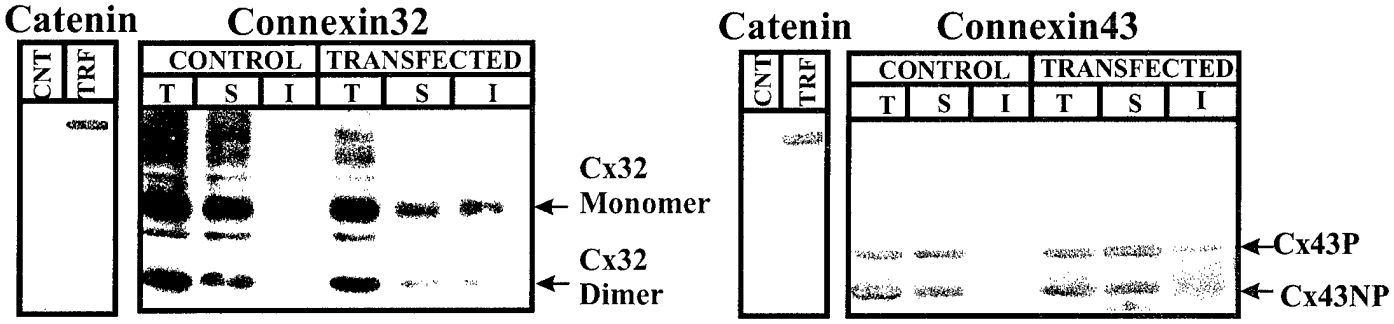


Govindarajan et al. Figure 6

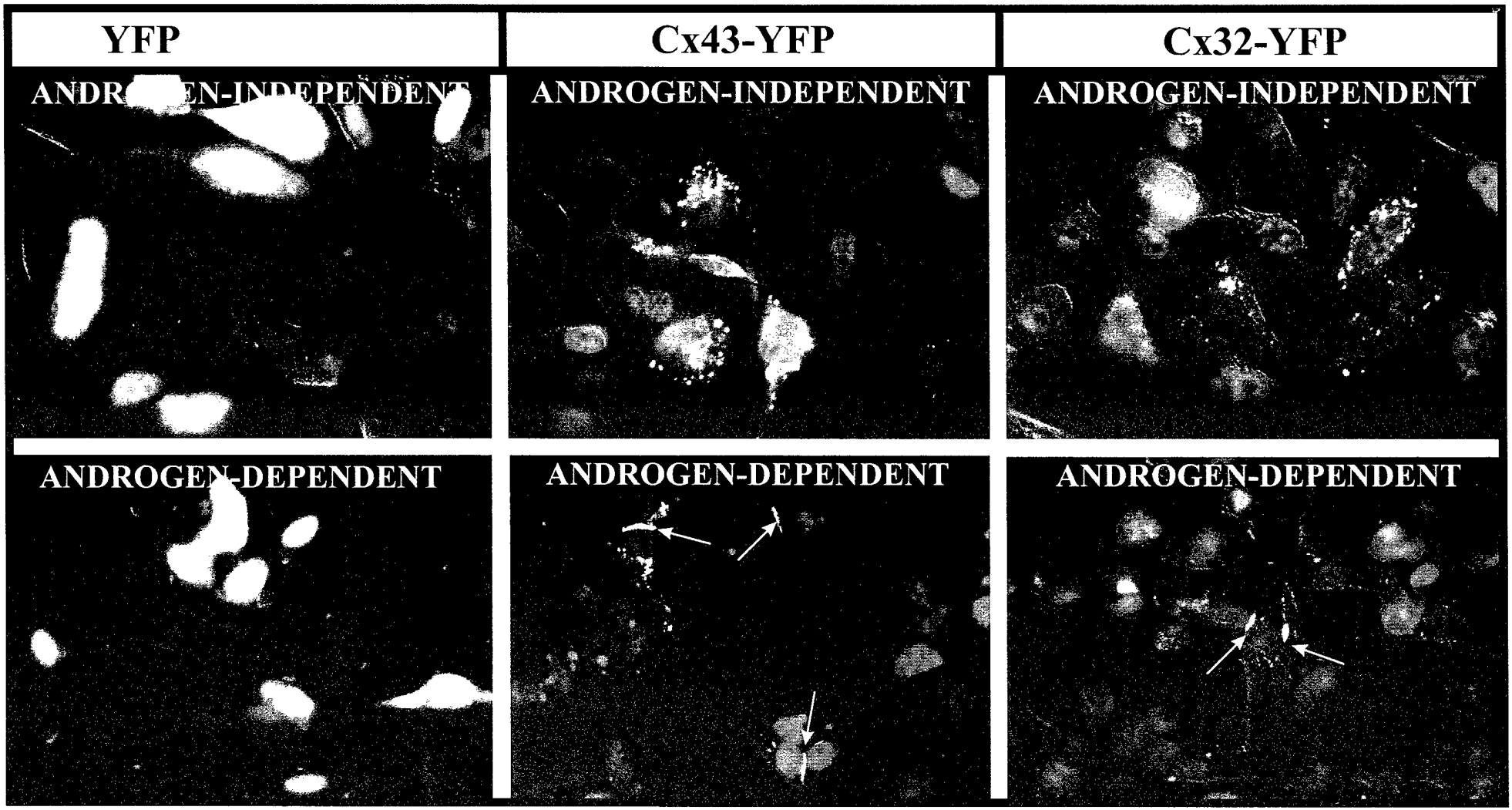


Govindarajan et al. Figure 8





Govindarajan et al. Figure 9



Govindarajan et al. Figure 10